

SDI at the Geneva summit

The United States has a month to work out what line to take on the Strategic Defense Initiative at next month's summit. It should unpack the proposal, and be willing to negotiate.

PROPAGANDA is not always lies. That is what the US administration should remember when brushing off the present flurry of Soviet protests about the plan to build a US defence against ballistic missiles, called the Strategic Defense Initiative (SDI). Obviously the welter of talk is meant as preparation for the summit meeting between President Ronald Reagan and Mr Mikhail Gorbachev in November, and so qualifies as propaganda of a kind. The difficulty, for the United States, is that the Soviet arguments are bound to seem reasonable to third parties and perhaps even to some who pay taxes in the United States. Who would not trade the uncertain and even risky outcome of SDI for the 40 per cent reduction of strategic missiles that Soviet spokesmen are hinting at? And the Soviet Union cannot have known in advance that its case would be strengthened by last week's report from the US Congress's Office of Technology Assessment (see *Nature* 26 September, p. 276). The administration is boxed in.

It is in everybody's interest that the knot should be untangled before November. Three questions arise. What is SDI and will it function as advertised? Could its products be deployed without aggravating present dangers? And, if there are likely to be benefits, what would they be worth as trades? The Office of Technology Assessment (OTA) says no to the first two questions (of which the second is the more important). The third has been only inadequately discussed so far.

The feasibility of SDI, always disputed, remains in doubt. Nothing has happened in the past year to clarify the workings of a possible system, but that is no surprise. So it remains that the only elements of a comprehensive defence against ballistic missiles to have been demonstrated are at the beginning and the end of a rocket trajectory. The US Vela satellites have probably shown the military during the past twenty years that it would be possible to design a system of infrared sensors based in satellites to detect hostile missiles rising through the atmosphere, while the anti-missile rockets around Moscow are a sign that terminal defence is also feasible, but at ruinous cost. The immediate difficulty is that these elements in a comprehensive defence would, if deployed, conflict with the Anti-Ballistic Missile (ABM) Treaty of 1972, the first with the spirit and the second with the letter. But a better early-warning system coupled with some terminal defence is not what SDI is all about.

Layered defence

Layered defence is, rather, SDI's new slogan. The idea is that there are several stages in the trajectory of a hostile missile when it could (on paper) be destroyed by different means. Ground-based rockets aimed at incoming warheads are the conventional stage of missile defence, but SDI counts most of all on means for attacking missiles as they are rising through the atmosphere, when they are most conspicuous and before they have split into

separate warheads (plus decoys). Then, the calculation runs, no single layer in the defence has to be totally impenetrable; it will suffice that each of them should have means of destroying missiles with substantial probability. Sixty-six per cent success in each of five layers would mean that less than 0.5 per cent of the missiles in a hostile force would reach their targets, which would make a first-strike impracticable unless missile numbers were enormously increased. OTA is a little unkind not to acknowledge that US spokesmen (but not the President) have been saying this for the past two years. But it seems constantly to be forgotten that even a successful SDI will not yield a layered system providing the kind of defence in depth required. If the programme is at all successful, it is most likely in the first instance to consist of a necessarily leaky means of destroying some missiles while still within the atmosphere — coupled with a promise that other layers will follow later. But the pressure to deploy, after all that money had been spent, would not be easily resisted. But even the US administration admits that a half-baked defence would be a danger.

The same dangers would obtain, and for a very long time, even if SDI should turn out to be a great success. The array of satellites that would have to be deployed, carrying different kinds of destructive instruments, could not be put in place except over a period of several years. The essence of OTA's conclusion is that the job could only be done with the agreement of the Soviet Union, which is why its report argues that the agreement had better be negotiated now, before all that money has been spent, for one thing.

Risks

What are the risks? President Reagan's aphorism (said to have begun with the Secretary of Defense, Mr Caspar Weinberger) is that "we aim to destroy missiles, not people". The implicit assertion is that the assurance that inanimate objects can be put out of action must surely be preferable to a strategy based on the threat of killing people. The flaw in the argument is that it takes no account of the Soviet Union's perception of what success for SDI would imply. An effective defence against strategic missiles would necessarily seem to the Soviet Union as a one-sided licence to engage in military adventures (Berlin, Hungary, Czechoslovakia, Iran or latter-day versions of those earlier trouble-spots) without fear of nuclear retaliation. It would also seem a one-sided licence to launch a first strike. But no protestations of peaceful intentions by the United States, however genuine, could set such fears at rest. These were the arguments that fifteen years ago persuaded the United States and the Soviet Union to sign and ratify the ABM Treaty. Technology may have advanced since then, but the principle remains. And while it may be only partly credible that the Soviet Union might be goaded into launching a pre-emptive strike against the US strategic force if there ever seemed a danger that a full-blown missile defence would emerge from SDI, it is certain that the long and uncertain process of deployment would be a powerful means of losing friends and allies.

So what should Mr Reagan be prepared to trade in November? The administration is right on one point, that arms control agreements that seek to ban research of certain kinds are not merely worse than useless but dangerous: because they cannot

Biological manuscripts

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be verified, those who stick by the rules are at a disadvantage. President Reagan has insisted for the past several weeks that SDI, as a research programme, is not a negotiating counter. He should stick to that, letting the US Congress ultimately decide the scale on which the research programme is carried out. (Congress will probably be even meaner in future years than the likely cut of more than a third in the present budget seems to suggest.)

The early-warning part of a missile defence is in a different case. The ABM Treaty restricts the right of the two parties to build early-warning radars except at the periphery of their territory (when they must look outwards) for fear that they might be used to extend existing terminal defences (of which the Moscow system is the only one extant). But if it is possible to build effective means of telling when strategic missiles leave their launching pads, the present strategic balance would not be undermined but, rather, strengthened. More warning, if only an extra 20 minutes, would allow more effective retaliation, perhaps before the first warheads had reached their targets. So both sides have an interest in effective early warning, and the ABM treaty should be amended to allow for that. It is by no means fanciful to suggest that each side should give the other a chance to monitor (and comment upon) the data collected by the warning satellites, along the lines proposed in the 1980 draft of a comprehensive test-ban treaty (which came to nothing). Then there would be a chance that false warnings would be screened out.

So Mr Reagan's best strategy on SDI is to stick with the assertion that research programmes cannot be codified by treaties, but to offer to unpackage SDI, putting early-warning systems at the top of the list and offering to concentrate the rest of the programme on the layers most likely to yield quick results, conspicuously the boost-phase part of the interception problem. (That, in any case, is probably how the work will go.) It is not realistic to suggest, as some have done, that the research itself might be carried out collaboratively between the Soviet Union and the United States; fears that the other side would keep back interesting tit-bits of information would be justified. But if there is now to be an agreement to restrict the numbers of strategic missiles below the quotas laid down in the unratified SALT II treaty, it should not be beyond the wit of those assembled at Geneva to agree that SDI would be deployed only stage by stage, and only after a negotiation in which the quotas of strategic missiles were adjusted appropriately.

Two further qualifications arise, with the question of anti-satellite weapons inseparable from that of SDI. The United States last month carried out the second successful test of such a weapon, justifying the breaking of the self-imposed Soviet moratorium by pleading disadvantage. But what are such weapons for? The obvious applications are the destruction of surveillance satellites and of the kinds of telecommunications that might be necessary to give early warning. But if there are ever satellite-based missile defences, they will also be vulnerable.

It is hard to see how any of these functions could benefit a strategic power with no interest in making a pre-emptive strike against the other. In short, an agreement not to test and deploy anti-satellite weapons should be in everybody's interest. The difficulty, real enough, is that of verification. The US administration should say as much.

The way in which the United States talks about the impending summit is also important. Mr Gorbachev has had the better press so far because he and his associates have been saying the same things over and over again. The United States administration, endearingly open as it is, speaks with a dozen voices, not one of which could be mistaken for a conviction that the November summit is an opportunity to make substantial progress on the issues of arms control which have been log-jammed now for close on three years. So much is clear, even though it would have better for people's peace of mind (and also more likely to lead to a constructive meeting) if less were to be expected of a two-day encounter between two people who have never met before. □

Money earthquakes

Mexico's tragic earthquake could cause nasty after-shocks for the banks.

It will be a cruel irony if the earthquake that killed more than 5,000 people in Mexico City two weeks ago should also bring the international banking system to another crisis, but that is how events may turn out. The bankers and their governments are doing very little to help avoid the trouble. The facts are now well-known. The nations of Latin America have accumulated foreign debts to the international banking system which amount to some \$350,000 million, of which Mexico owes nearly \$100,000 million. The origins of this strange state of affairs are to be found in the 1970s, when the newly-rich oil suppliers lent their surplus dollars to the commercial banks of the developed world, which in turn recycled them (in part) to the countries in need of cash (in order to buy oil) but which could not attract sufficient funds on their own account. Mexico is a particularly sad case for, while an oil-producer itself, it is also the largest debtor.

The accumulated debts of Latin America have been a widely publicized headache for the bankers for at least the past three years. Their difficulty is that if too much of what they have lent turns out to consist of bad debts, they will make losses instead of profits and their capital base will be eroded and their capacity to lend fresh funds to productive enterprises will be constrained, to everybody's disadvantage. The danger of old-fashioned "runs" on the banks as in the 1930s are less now than they used to be but are not entirely negligible. That at least is how it seemed three years ago.

Since 1982, however, the ever-more ingenious bankers have almost made the problem disappear. Payments on loans have been "rescheduled", countries unable even to pay the interest have been lent fresh funds, but the arrangements have all been backed up by a tough agreement with the International Monetary Fund whose effect has been to provide access to a modest amount of credit provided by other governments, but which has required that the recipients should take draconian measures to control inflation and internal costs so as to export their way out of trouble.

Even before the earthquake, the Latin American countries were resentfully grumbling that the arrangements made in the past few years are too onerous. Mexico itself, on the eve of the earthquake, was thought to have failed to meet the conditions of the International Monetary Fund, so that it would have been disqualified from further access to credit from that source. Since the opening of this year's session of the United Nations General Assembly last week, the argument that there must be a new global solution of the debt problem has been repeated several times. So, indeed, it should, but not by the means most attractive to the debtor nations, that of forgiving past debts and starting with a clean slate.

High interest

A large part of the trouble is that interest rates have remained obdurately high during the past five years, with the consequence that interest accumulates on outstanding loans at a much faster rate than when they were originally contracted. Worse still, economic growth in the industrialized countries has not revived as quickly as it might have done, with the result that exports from Latin America have not grown as quickly as was hoped or expected, although Brazil and Argentina have recently done well in selling to the United States. Mexico again has been the black sheep. Now the difficulties which confront Mexico are physical and enormous. The costs of reconstruction will be huge, and not covered by the present wave of generosity. No doubt the bankers will think first of stretching out the loans once more, further increasing the ratio of accumulated interest to the original debt. Mexico will have other Latin American states on its side if it decides to jib at the prospect. Then we could all share Mexico's troubles. □

AIDS

US and French institutes in patents struggle

TENSION is rising on both sides of the Atlantic over the matter of patents and royalties on diagnostic kits for acquired immune deficiency syndrome (AIDS). The dispute centres on the Institut Pasteur in Paris and the National Cancer Institute in Bethesda, with the Pasteur as the aggrieved party.

Luc Montagnier, the French co-discoverer of the virus that causes AIDS, believes that the royalties earned on patents for AIDS diagnostic kits should be divided equally between the Pasteur and the United States Department of Health, whose National Cancer Institute researcher Robert Gallo also isolated AIDS virus (see page 395). "That would correspond to the two scientific contributions", Montagnier said last week.

Matters will come to a head this month when the Food and Drugs Administration is expected to approve the Pasteur test, to be marketed in the United States by the Seattle-based Genetic Systems Inc. The problem is that whereas Gallo has a US patent on the AIDS test kits the Institut Pasteur does not — even though the Pasteur filed its patent application months before the US Department of Health. In the present state of the patenting argument, the Pasteur will be obliged to pay royalties to the Department of Health on the basis of the Gallo patent, but "that would be ridiculous", Montagnier claims. Robert Nowinski, chairman of Genetic Systems, is clear on the point: "Genetic Systems does not anticipate paying royalties to the US government."

Hence there have been increasingly urgent attempts by the director of the Institut Pasteur, Raymond Dedonder, to resolve the patent issue, in particular by a possible attachment of the Pasteur's name to the existing Gallo patent. A letter from US Secretary of State for Health Margaret Heckler to Dedonder demanding further evidence of the French claim before anything could be settled led to an explosion in Paris last month — with Dedonder threatening publically to go to law against the US government "to claim our rights". These would be a share — Montagnier has said half, others have suggested more — of the \$100–150 million in royalties that are expected to arise from the use of AIDS tests. For the Pasteur, which is a private institution, this would be no mean sum, equivalent to many times its annual budget.

As for the claim itself, Montagnier points out that HTLV-III (human T-lymphotropic virus III, Gallo's) and LAV (lymphadenopathy-associated virus, Montagnier's) are "extremely similar by any

criteria". The viral sequences differ by only 1.5 per cent and an independent US group will publish evidence that there is no difference between the calculated restriction maps of HTLV-III and LAV, whereas there are differences among all the maps of nine other AIDS virus isolates. Montagnier sent Gallo his viral isolate in July 1983, he has claimed (*Nature* **310**, 466; 1984), and again later in September that year. Montagnier filed his US patent application for AIDS antibody tests based on enzyme-linked immunosorbent assay (ELISA), using purified viral extracts, in December 1983, but Gallo filed his similar application only in April 1984. Gallo's application was granted in May 1985; but Montagnier is still waiting.

The Pasteur patent application emphasizes a core protein (p25) of the virus, rather than an envelope protein, to stress differences between HTLV-III and the HTLV-I and -II viruses, says Montagnier, "but the virus extract should include all viral proteins". Gallo's patent, in contrast, emphasizes the p41 envelope protein. If the two tests indeed sought different antibodies, one possible outcome would be two separate patents, but it now seems they are too similar for that to be feasible. The US Patent Office will probably be obliged to declare an "interference period" — which may last a year or more — during which time neither side would be permitted to take legal action against the other and no royalties would be paid by either party.

Although the existing tests licensed on the US market have the advantage of priority, Genetic Systems boasts that in a recent clinical trial in Australia the Pasteur test achieved 100 per cent sensitivity and specificity on 6,000 samples from 1,000 patients, a clear improvement on any of the existing tests. The difference is ascribed to the production of the virus in a cell line that does not produce contaminating HLA antigens that can lead to false positives.

Does Montagnier feel surprised at the delay in granting the patent? "What is more surprising is the speed with which Gallo's patent was issued", he says. Nevertheless, Montagnier emphasized, "I'm very confident in the American administration doing their duty without political interference — and that our patent will be accepted very soon." The natural justice of the matter was that "we've made a good contribution to this problem, and Gallo has made a good contribution, and so we should share the rights and the royalties."

Robert Walgate & Tim Beardsley

Biotechnology

New plans for regulation

Washington

THE White House's working group on biotechnology policy has changed its tune over how the federal government ought to plan regulation in the area. Earlier plans for a Biotechnology Science Board made up of scientific advisers from each of the five relevant government agencies have been shelved, and current thinking favours instead a committee of federal officials at assistant secretary or under-secretary level.

At the end of last year, in a plan that was far from universally popular, the Cabinet Council Working Group on Biotechnology suggested establishing a scientific advisory group analogous to the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) within each of four agencies — the Food and Drug Administration, the Department of Agriculture, the National Science Foundation (NSF) and the Environmental Protection Agency. Representatives from each of these four committees and from RAC itself would comprise the Biotechnology Science Board. Critics of the plan feared that the new board would merely duplicate on a more elevated plane, and eventually supplant, RAC and its equivalents in other agencies, and many researchers felt that RAC's accumulated expertise could be protected.

The committee now proposed would operate under the Federal Co-ordinating Council on Science, Engineering and Technology, which is chaired by George Keyworth, the President's science adviser. It would restrict itself to defining what the scientific issues should be for each agency and would not make decisions about individual proposals.

As currently envisaged, the new committee would include two representatives from each agency. *Ad hoc* subcommittees of outside advisers could be established as necessary to handle specialized questions. The committee would be chaired alternately by NIH and NSF, and the whole realm of biotechnology would be within its purview.

The new plans were outlined at a recent RAC meeting by Bernadine Healy, until last week chairman of the Cabinet Council Working Group on Biotechnology. They will be formally published next month. One issue that has already given rise to concern is how accessible, or at least visible, to the public the new committee will be. According to those working on the plan there will be public access to the individual RAC-equivalents, and some provision will also be made for access to the full committee, but the exact details have yet to be settled.

Tim Beardsley

Environment

Bankers under pressure

Washington

IN an unusual alliance between Congress and the administration, the US Senate and the Treasury Department are together pressurizing the multinational development banks to improve environmental safeguards for their loan programmes to developing countries by, for example, "improving organizational capacity" and "more stringently conditional" loan disbursement. The banks are also criticized for their reluctance to discuss their efforts in this area with environmentalist groups in the United States and in the countries concerned.

The good intentions of the banks are not in question, but environmental organizations have successfully persuaded Congress and the Treasury that guidelines set by the banks are often not being properly followed, resulting in massive damage. Congress, in the shape of Senator Robert Kasten's (Republican, Wisconsin) foreign operations appropriations subcommittee, is determined that the banks should make "institutional changes" in their organizations to prevent disasters such as the Polonoreste project, a \$434 million loan by the World Bank to build a road through the Amazon rain forest of Brazil that caused massive deforestation of an area the size of Italy. The project was halted earlier this year as a result of continuous campaigning by environmental groups and subsequent congressional pressure, but Kasten fears that there are other projects where there is a "blatant disregard" for environmental protection and "insensitivity to native peoples".

One such project is the Narmada river basin dam in India, to be financed by the World Bank. Inadequate resettlement plans for more than a million people from 250 villages and the bureaucracy of state government has caused bitterness and "does not inspire optimism" in the bank, says David Maybury-Lewis, professor of anthropology at Harvard University. Maybury-Lewis singles out projects in Paraguay and the Philippines as evidence for "bitter and pauperizing consequences of bank-financed relocation" and attacks the bank's recent agreement to finance a huge Indonesian transmigration scheme.

Kasten's subcommittee, which oversees funding of the development banks, last year instructed the World Bank to provide a high-level environmental review, to stop funding large-scale capital-intensive projects and instead to finance small-scale environmentally beneficial schemes and to ensure that non-governmental groups in the countries involved are consulted. The Treasury, directed to work with the bank, called progress since then "modest" at recent hearings of the subcommittee; the World Bank has not publicly responded to any of last year's requests.

Therefore, Kasten is likely to introduce "very strong language" into the appropriation for the next fiscal year. One possibility is that the US directors of the bank will be asked to hold a special board meeting and report to Congress on what actions they are taking to ensure that their environmental guidelines are being met.

The World Bank spent \$800 million last fiscal year (5 per cent of its total lending) on environmental projects, mainly improving water supplies, sanitation and reforestation schemes. The bank provided a detailed discussion of environmental

issues in its annual report as evidence for its "great concern" about the detrimental impact of some projects. Bruce Rich of the Natural Resources Development Organization said that although the bank describes the problems well, it does not say how it is going to avoid contributing further to them.

The bank is unlikely to be able to maintain its aloof stance from its critics for very much longer. Although more than 100 countries own the bank, the United States is the single largest shareholder and can exert "considerable leverage" on the bank. Specific projects may soon be under more severe public scrutiny before development banks can provide the capital to implement them.

Maxine Clarke

EEC chemicals

Controversy over controls

Brussels

DIFFICULT discussions on controversial EEC legislation designed to tighten up controls on trade in dangerous substances with countries outside the European Community have reached an impasse at departmental level. The problem is now to be left to the 14 members of the European Commission when they meet in October in the hope that European Commissioners might achieve what plain reasoning has obviously not.

Until now, there have been no legal restrictions on EEC companies that wish to export to the developing countries chemicals whose use is restricted or banned in the Community. But a new and very unofficial EEC proposal would require that before being able to export certain pesticides and other potentially dangerous substances (such as polychlorinated biphenyls, polychloroterphenyls and asbestos), exporters would have to notify the government of the importing country and inform it of the hazardous status of the substance and the dangers involved in its use. The government would then have up to ninety days to refuse import permission.

Export authorization would be granted by the European Commission for a six-month renewable period for a specific volume only. Subsequent exports would have to receive the approval of the designated authority set up by the EEC in each of the member states to keep an eye on trade in dangerous substances. Each shipment would also have to carry details about the toxicity and risks of the product.

Not surprisingly, the proposals are strongly contested by those with industrial interests. Among the objections raised is the likely impracticability of the scheme in developing countries which often lack the administrative infrastructure necessary to respond within the given time. Industrialists fear that the only result will be a 90-day delivery penalty for EEC-based exporters, who would face unfair competi-

tion and suffer a loss of markets to non-EEC companies (in countries such as Switzerland) out of range of such laws.

If adopted, the EEC proposal would constitute a *volte-face* on the part of the Commission. Despite pressure two years ago from the Dutch and Greek environment ministers, the then Environment Commissioner, Karl-Heinz Narjes, who now has the industry portfolio, tried to put the lid on the debate by insisting that restrictions have been urged from the European Parliament, countries within the Community, and from further afield.

Notification and information procedures have already been adopted by the Organization for Economic Cooperation and Development and the United Nations Environmental Program but these involve prior notification, not the more far reaching concept of prior consent. (The current EEC draft proposal gets halfway there by suggesting a system of prior refusal.) The Food and Agricultural Organization's code of conduct for pesticides, which comes up for adoption in November, originally contained the notion of prior informed consent but has since been watered down.

Even though the EEC proposal goes further than anything adopted to date by other international fora, if adopted in its present form it would not be going far enough for a coalition of international non-governmental organizations known as 'CADE' (Coalition Against Dangerous Exports). Last week CADE published a report detailing the impact on developing countries of the double standards practised by the European Community. It reveals, for example, a current bid for tenders under the European Development Fund for companies prepared to export 40,000 litres of endrin and 60,000 kilograms of aldrin (both very dangerous pesticides) for application on a coffee plantation on the Ivory Coast. The Community debate in coming months promises to be a hot one.

Anna Lubinska

Extraterrestrial intelligence

Is there anyone out there?

Washington

A NEW search for extraterrestrial intelligence was launched last week at Harvard, Massachusetts. A multi-million channel receiver operated by the Planetary Society will search 8.4 million radio channels for possible signals from an alien civilization, 64 times more than the society's existing Project Sentinel. Construction of the new receiver, called META (for Megachannel Extraterrestrial Array) was financed by a £100,000 gift from Steven Spielberg, director of "ET", the blockbuster movie about the adventures of an alien stranded on Earth.

The antenna to be used by META is an 84-foot diameter dish at Harvard University's Oak Ridge Observatory which was due to be dismantled until the Planetary Society stepped in. The META receiver was designed and built by Paul Horowitz of Harvard University, and has been constructed for a knockdown price because it searches only selected "magic frequencies" — frequencies corresponding to emission frequencies of the most abundant atoms or radicals in the Universe. According to the argument, an alien civilization seeking to broadcast its presence might choose a "magic frequency" to send its messages. META will look initially for ultra-narrow bandwidth signals (using a

by members. Other searches for extraterrestrial intelligence are under way in the Soviet Union and elsewhere. Ohio State University's "Bigear" radio telescope, which supports one search, has for the past two years been under threat of demolition in order to make way for a golf course; last month the threat was averted, however, and the telescope now looks set

to continue operations for at least another 10 years. And despite severe budget restrictions, the National Aeronautics and Space Administration (NASA) has been able to spend around \$1.5 million a year building a receiver that, when it comes into operation in 1988, will search all frequencies that are reasonable for interstellar communication, from 1 to 10 GHz. But NASA's search, unlike that of Horowitz's instrument, will look for pulsed signals and examine individual nearby Sun-like stars.

Tim Beardsley

Primate research

NIH withdraws support

Washington

THE University of Pennsylvania last week banned primate research and reprimanded Thomas Gennarelli and Thomas Langfitt of the head injury research laboratory, but rejected allegations of cruel and inhumane treatment of animals. Representatives of People for Ethical Treatment of Animals, who last year stole researchers' videotapes, called the university's decision "most responsible". In studies that were carried out in collaboration with the University of Glasgow, in Scotland, the laboratory has been using primates to model the effects of brain damage in humans. Financial support for the research has now been withdrawn by the National Institutes of Health (NIH) pending a full enquiry, and the Department of Agriculture (DoA) is suing the laboratory for violation of the Animal Welfare Act.

The university has decided that NIH guidelines for animal research were not met in the supervision and training of staff or in sanitation and postoperative care of

animals. These findings are considered "particularly troublesome" in view of worries already expressed by the university's animal care committee as long ago as 1982. Research will not be resumed until the committee, which is being reconstituted according to new public health guidelines, NIH and DoA, are satisfied.

Gennarelli and Langfitt said last week that all research programmes at the laboratory have been jeopardized as a result of the ban on primate projects, which represent less than a quarter of the laboratory's work (the rest is computer simulation and modelling). Gennarelli and Langfitt acknowledge some "minor infractions" but refuse to abandon the hope of resuming their work on injuries to primates. Although the university has reaffirmed its "strong support for the use of animals in biomedical research", Gennarelli and Langfitt think that permission to continue their "controversial" research will be withheld "for a long time" because of public protests.

Maxine Clarke



0.04 Hz channel width) in the noisy 1,420 MHz region, corresponding to a major emission frequency of hydrogen.

Supporters of the "magic frequency" strategy argue further that an intelligent alien might correct the frequency of its broadcast for its own motion relative either to the Galaxy or to the Universe as a whole, so that a similarly intelligent listener, by compensating for its motion, could eliminate Doppler shift. The Earth's motion relative to the rest frame of the Universe and of our Galaxy is now known, and META will be the first receiver to search the 1,420 MHz hydrogen band in these rest frames. A major emission frequency of the hydroxyl radical at 1,700 MHz might also be examined later.

Spielberg's gift was made on the condition that the Planetary Society continue to support operation of META for several years, which it plans to do from donations

US "big science" looks abroad

Washington

THE US National Research Council (NRC) is contemplating launching a major study on whether increased international collaboration on "big science" would be a desirable means of increasing funds for major science and technology facilities. A blue-ribbon panel chaired by Frederick Seitz, a past president of the National Academy of Sciences, and Ralph Gomory, of IBM, spent two days last week discussing the question and will shortly make a recommendation for a formal study to NRC's Commission of Physical Sciences, Mathematics and Resources.

The study was inspired by growing concern that, with attempts to limit the national budget deficit a priority for Congress and the spiralling cost of the large facilities now required by physicists, some planned projects might face long delays unless other sources of funds are found. The proposed Superconducting Supercollider, which would probably cost more than

\$3,000 million, is an obvious case in point. Although international partners are unlikely at this stage to be interested in sharing construction costs, they might be willing to supply instruments for the collider, which may themselves cost hundreds of millions of dollars, according to NRC's Herbert Freidman.

Space research is another area with significant potential for international collaboration, according to Freidman; the planned International Solar Terrestrial Physics Program might become a model for future collaboration. Cooperation over the planned space station and space-based interferometry are also being considered, as are areas that might be included in the study.

The proposed study has yet to be formally approved by several layers of NRC bureaucracy, which could take months. After that, the search will be on for agencies willing to put up the money needed to pay for it.

Tim Beardsley

German molecular biology

New centre is launched

Heidelberg

DESPITE an attempt to burn it down before it was occupied, and still under makeshift leadership, the Centre for Molecular Biology of the University of Heidelberg (ZMBH) has begun to fill up and is heading confidently for its official inauguration in November.

ZMBH has its origins in the decision of the German chemical company Hoechst in 1982 to invest US\$ 50 million into a new department of molecular biology at Massachusetts General Hospital in Boston. Stunned by both the sum of money and its export to the United States, University of Heidelberg molecular biologists persuaded BASF, best known for cassette tapes, to provide the seed money for ZMBH. The state of Baden Württemberg then chipped in with a building and salaries for 30 technical and administrative staff and the federal ministry of science and technology (BMFT) provided financial support to ZMBH as the first of four national gene centres.

Built in the shadow of the German Cancer Research Centre, which has about 60 per cent of its 1,200 staff in permanent posts, ZMBH with 200 staff will give tenure only to eight or so senior scientists, not all of whom have yet been recruited. Though full professors of the university, their teaching load will be a quarter of the standard eight hours a week, which is possible because ZMBH has the status of a central institute of the university, like the botanical garden. They will, nonetheless, take both graduate and postgraduate students.

Research funds will come from three core grants, the biggest one of which has been approved in principle by BMFT and will be worth at least DM6 million for each of the next four years. The other two grants are in the form of *Sonderforschungsbereich* support from the German Research Society (DFG). One grant, for neuroscience, has been approved. The other, for gene expression, is expected to be approved soon following a site visit two weeks ago.

BASF's contribution of an annual DM1 million for ten years seems in part to be slush money. It is likely, for example, to help provide a sufficient salary to lure to ZMBH a senior German scientist in a leading US biotechnology company. In return for their support, BASF gains only the right to attach two of its researchers to ZMBH. Merck (of Darmstadt), who have recently agreed to provide a smaller sum of money, will be allowed one attached scientist in return.

Despite various ambitions, there is no director of ZMBH and it is not clear if one will emerge. Initial attempts to sort the matter out were confounded by possible conflicts of interest when some of the

candidates founded a biotechnology company (there is now a rule that industrial involvement is acceptable as long as it does not include membership of an executive board). At present there is a directorate of five senior staff with Professor Heinz Schaller as their elected "speaker". But with the imminent arrival of Hermann Bujard, one of the initiators of ZMBH and presently at Hoffman-LaRoche in Basle, it seems likely that the directorate will be slimmed down and that Bujard, or perhaps Schaller, will head it.

Above the directorate is the rector of the university who is assisted by a scientific advisory board. The board, which is international, advises on senior appointments and the scientific programme of ZMBH.

As long as there is no repeat of the bungled attempt to burn down the building — a fuse foiled the unknown arsonists, who seem to have been driven by a gene phobia — ZMBH should be a good showplace for the University of Heidelberg as it celebrates its 500th anniversary next year.

Peter Newmark

China chases share in Eureka

Beijing

THE People's Republic of China seems anxious to play some part in Eureka, the French inspired programme of collaborative research and development in high technology. So much became clear at the end of last week, during meetings between a French delegation led by M. Hubert Curien, the minister of research in Paris, and officials of the Chinese government.

Members of the French delegation said they had been surprised at the way in which the Chinese side had brought up the issue of participation early in the talks, which among other things covered collaboration in biotechnology. Precisely how China might play a part in Eureka was by no means clear but in circumstances in which the French enthusiasm has not been matched by that of other European governments, the chances are that some way will be found.

Meanwhile, Chinese researchers are intrigued that one of them, Professor Guang Zhao, was promoted to become a member of the Central Committee of the Chinese communist party at last week's national meeting, that at which veteran members of several party organs were replaced by somewhat younger people.

Professor Zhao, once head of the Beijing Institute of Theoretical Physics, is 56, and is believed to be the intended successor of Professor Lu Xiang, now president of the Chinese Academy of Science.

John Maddox

Rural India

Longer life for thatched roof

New Delhi

INDIAN scientists engaged in improving the lot of the rural poor have found a simple, cheap and effective method for extending the life of the perishable coconut leaf thatch and making it fireproof. Coconut leaf (with a world production of 42 million tonnes) is a major roofing material in the tropics, and in India alone some 38 million houses are thatched, as well as thousands of cinemas and schools. The thatched roofs normally need replacement every year, but their life span has now been extended to four years by treating the thatch with chemicals that are neither washed away by rain nor degraded in sunlight.

"The use of treated thatch will lead to enormous savings in materials and labour", says Dr C.K.S. Pillai, a polymer chemist at the Regional Research Laboratory (RRL) in Trivandrum, in the state of Kerala. During a five-year research project, Pillai and his team found that coconut leaves deteriorate due to rain, alternate wet-and-dry cycles and more importantly due to attack by fungi entering through the stomatal openings of the leaves. Five types of fungi were isolated from decayed thatch and were subsequently identified by the Commonwealth Mycological Institute in London. So Pillai attacked the problem by coating the thatch with a combination of fungicide and a water repellent.

In the process recommended by RRL, copper sulphate is used as a fungicide because it is cheap. The chemical is dissolved in water and sprayed on both sides of the thatch, or the thatch is dipped in copper sulphate solution and dried. The dried thatch is then sprayed on both sides with a liquid extracted from the shell of cashew nuts (CNSL) and mixed with kerosene. CNSL, a by-product of the cashew nut industry, is an excellent water repellent besides being cheap. Trials conducted by Pillai have shown that roofs made of treated thatch survive for four years while untreated roofs, under similar conditions, collapse in 12 months.

Chemically treated thatches are now being used on a large scale in Mitrani Ketan village in Kerala, and other villages are catching up with this new technology.

Although the copper sulphate/CNSL combination extends roof life, it does not provide immunity to fire. To make the thatch fireproof, the RRL team converted CNSL into its phosphorylated derivative — an excellent fire retardant — by heating CNSL with phosphoric acid. A coating of copper sulphate followed by spraying of phosphorylated-CNSL is recommended by RRL to give added life to thatch as well as to protect it from fire.

Attempts to extend the life of coconut leaf thatch have been going on for over a decade in several laboratories across the world. At least nine methods have emerged for extending thatch life and 12 chemical treatments have been suggested for making it fireproof. Pillai says the chemicals used so far are either costly or a health hazard or leached by rain. The RRL recipe is claimed to be cheap, safe and nonleachable.

As well as India, the RRL process may benefit Sri Lanka and the Polynesian islands where coconut leaf is the main roofing material in rural homes. Longer life for thatched roofs would result in surplus coconut leaves which, according to RRL, could be used to make newer products. Pillai and his team have already made brown paper from the pulp of the leaves, and laminated products such as table tops and door panels directly from the leaves. "The laminates are made by plaiting the leaflets into sheets and using these sheets as plys as in the case of plywood", says Pillai.

K.S. Jayaraman

US biological weapons

Protests over US Army lab

Washington

THE latest attempts by lobbying groups to persuade Congress to regulate the military use of biological research has centred on the US Army's "aerosol test facility" at Dugway, Utah.

Fourteen groups, coordinated by the Committee for Responsible Genetics, are seeking a ban on all classified research and development "aimed at producing new or altered biological agents with properties useful for biological and chemical warfare". They also want an investigation into allegations that the Soviet Union and its allies are developing biological weapons, incorporation of the 1972 Biological Weapons Convention into US domestic law and a complete halt to the proposed aerosol testing facility.

Congress has approved a \$300 million modernization of the Dugway Proving Ground, the Army's primary test site for biological and chemical research. However, the controversial part of the project is the proposed special testing laboratory for handling "toxic biological aerosol agents", and construction of the laboratory has been delayed by a court injunction which will remain in force until the Army produces an "adequate" environmental assessment.

The Army has responded by stating that it will prepare an "environmental impact statement", a much more detailed study than required by law, and will contract out the work to an independent organization in an attempt to avoid suggestions of bias. An Army spokesman stressed that the

Acid rain

Upwind versus downwind

LESS affected countries relegate acid rain issues to faraway forests: those more affected have so far failed to implement a policy designed to handle the chemical complexity and long-term effects of acid pollution. By drawing together worldwide data on the problem, a report published this week by the environmental pressure group Earthscan tries to bring the problem closer to home. It argues that acid pollution is rapidly spreading to the Third World, that downwind countries will place increasing pressure on upwind pollution sources, and that buildings and crops are as much damaged as trees and fish.

These data, according to the report, widen the scope of acid pollution. Not all acid deposition is wet, for example; one-third of sulphur emissions in the north-eastern United States is thought to be dry deposited, converted to acid on the surfaces of plants and buildings. Not all acid deposition is sulphur dioxide from fuel-burning plants; nitrogen oxide and ozone are among the culprits, and cars and fertilizers as much a problem as smoke stacks. Among the less industrialized countries, China faces the greatest risks, especially from inefficient combustion of low-quality coals; and Brazil, says the report, is one of the most polluted countries on Earth.

"Downwind countries like Norway, Sweden, Canada and Germany are simply not prepared to have their environment destroyed from abroad. There is already serious talk in Sweden of taking Britain to the International Court and in Norway of offering loans to Britain and Poland to finance anti-pollution measures at our power stations", says Jon Tinker, director of Earthscan.

The effects of acid pollution on trees and lakes have excited the most research, but the tangle of cause and effect has led to disagreement about the impact on the en-

vironment of such pollution. Last week, for example, Dr David Lonsdale, the plant pathologist of the Forestry Commission, claimed that the damage to Britain's beech woods was not caused by acid pollution. Resort areas dependent on their trees, however, such as West Germany's Black Forest, claim that there is enough evidence to justify governmental action.

The damage goes deeper than leaves on trees, according to the Earthscan report. As chemicals wash through the ground, the soil becomes more acidic. Soil under ice during the last Ice Age, including those in South America, Africa and southern Asia, are young in age and have less buffering capacity. Aluminium is a key prob-



lem. Normally tied to soil particles, chemical combination frees aluminium to damage root filaments and enter freshwater, where it has proven toxic throughout food chains. Lakes can be limed, but there are no data on how to treat soils, nor on how long they take to recover.

Elizabeth Collins

Acid Earth, by John McCormick, is published by International Institute for Environment and Development, London, and costs £3.95/\$6.25.

Dugway programme is "entirely defensive in orientation" and that there is no development of "delivery mechanisms" for biological agents planned anywhere in the Army's research programme.

Environmental and other organizations, however, fear that military biological research signals a "new spiral in the arms race". The validity of "defensive" biological research is questioned.

"Given the enormous natural variety of potentially infectious agents, together with the extraordinarily increased variation and novelty that genetic engineering technology provides, it is not possible to protect a population against biological warfare" Dr Jonathan King, professor of molecular biology at the Massachusetts Institute of Technology, told congressional representatives. And Dr Francis Boyle, professor of law at the University of Illinois, challenged the "Reagan administra-

tion's ... claims that the Soviets cannot be trusted with respect to the conclusion of future chemical and biological arms control agreements".

Dr Boyle argued that, in the absence of evidence for violations of biological weapons agreements, there is no need to support "recombinant DNA biological warfare research in the USA", although he recommended that Congress should "continue to monitor" the situation, as well as improving existing treaty arrangements.

Although the Army says that most of its evidence for Soviet involvement in biological warfare research comes from intelligence sources, one much-quoted example is the possible use of mycotoxins in "yellow rain" in South-East Asia. The Army refused to confirm or deny that mycotoxins would be used at Dugway.

Maxine Clarke

UK research

Neutron source inaugurated

WITH much fanfare, and in the presence of the prime minister Margaret Thatcher the Spallation Neutron Source (SNS) at the Rutherford Appleton Laboratory in Oxford was officially inaugurated this week. It is expected to be the last major facility constructed by Britain's Science and Engineering Research Council (SERC) for the foreseeable future.

Despite the delays and doubts that have afflicted its seven-year construction period, SNS is now acknowledged to be potentially the most powerful machine of its type in the world. Ebulliently, SERC's press office makes much of a quote that reportedly originated from the Argonne Laboratory, where there is a similar facility: "My God, they're going to blow us out of the water!"

Hitherto, British scientists wishing to make use of a neutron beam have depended heavily on the nuclear reactors of the Institut Laue-Langevin, Grenoble, a facility that is much oversubscribed. With a potential influx at the SNS of 4×10^{16} neutrons per second and an associated proton beam current twenty times greater than that available at the Argonne Laboratory, Britons would now seem to be well catered for. However, financial shortages have led SERC to negotiate with other countries, exchanging beam time with the provision of capital investment in the form of detectors, for example.

Another trade-off currently being investigated involves potential British use of the projected European Synchrotron Radiation Facility. Without such arrangements, SERC could only operate the SNS for about one third of the financial year 1986-87. As it is, the facility will close down — as far as users are concerned, at least — from Christmas until early April 1986, the start of the next financial year.

The principal users of SNS will be those wishing to use elastic and inelastic neutron scattering to probe solids and liquids. The neutrons are generated by the impact of a beam of protons on a uranium target. The protons arrive in two 100 ns bunches, 300 ns apart and repeated at 20 ms intervals, each pulse containing about 10^{13} protons. Having started off as negative hydrogen ions boosted to 70 MeV in a linear accelerator, the protons enter a synchrotron from which they impinge on the target with energies of 800 MeV or so. As the term "Spallation" indicates, the protons cause a greater number of neutrons to be ejected from the uranium, dissipating a quarter of a megawatt of heat in the process and leaving a highly radioactive target. Thus water cooling and remote handling techniques are essential.

Philip Campbell

UK environment

Labour's green charter

JOBS are a common political lure; less common is the claim that hundreds of thousands of them will result from proper environmental planning and research, as the UK Labour Party's Jobs and Industry Campaign promised last week at the launch of its environmental charter.

With proper planning, says Labour, economic growth and full employment can follow from such policies as product recycling, nuclear waste controls, reduction in pesticides, and protection of the countryside environment. Environmental research, says Dr David Clark, Labour environmental spokesman, is one source of new jobs as well as of the necessary data for planning. He criticized the recent slashing of the budget of the Natural Environment Research Council and of the Soil Survey, which faces a halving of its budget in April. Instead, he said, Britain should follow the example of West Germany, where 300,000 environmentally related jobs have been created.

Health problems around such plants as British Nuclear Fuel's Sellafield should be "relentlessly investigated" and the current moratorium on the dumping of radioactive wastes at sea should be continued. (The London Dumping Convention voted to suspend this method of disposal indefinitely last week but Britain voted against the resolution, which is not legally binding.) Such policies need a Department of the Environment with backbone says Labour, which wants the ministry to be a Department of Environmental Protection and Development. The new name is intended to signify a change in emphasis and inter-departmental clout.

Labour sees Britain's refusal to join the "30% Club" for acid emission reductions as "the most glaring example" of unmet international obligations concerning the environment. Britain is the largest producer of sulphur dioxide in Western Europe and yet no effort is being taken to retrofit power stations. "Not only is it morally wrong to pollute our neighbours' lakes and forests but it is economically stupid", according to Dr Clark.

In the countryside, "erosion of planning control" is manifested in the Forestry Commission, whose afforestation policies are "beyond control". Another offender, according to the Labour charter, is agriculture, "the largest subsidized industry in the country". Spurred by these subsidies, the industry produces "unsaleable surpluses of grain, butter and beef", while farmers are discouraged from conservation efforts and wildlife lose habitat.

Mrs Angela Rumbold, new Conservative Environment Minister, defended the government's policies by claiming that "management of the environment must be by the best practicable means . . . and clear both about the costs and benefits of taking action and about the risks of leaving things as they are".

She echoed the Labour charter by calling for more public disclosure of pollution data, particularly from nuclear facilities. She also pointed out the dangers Britain faces: "A 'cheap and dirty' image will be of no benefit to our industry at home or abroad." She did not, however, mention jobs as an ingredient of the government's "practical green policies".

Elizabeth Collins

IMAGE
UNAVAILABLE
FOR
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REASONS

Père David's deer which is to be re-introduced into China next spring. In 1900, most of the population was wiped out when they escaped from the Imperial Hunting Park near Beijing after its walls were damaged by a flood. The last wild specimen was shot in 1939 in the marshlands of the Yangtze delta where 30 deer from zoo populations will be placed in a new wildlife reserve. (Photograph courtesy of Zoological Society of London).

What future for fast reactors?

SIR—In their article¹ last year, Keepin and Wynne raised questions about the integrity of mathematical modelling in the energy study² by the International Institute for Applied Systems Analysis (IIASA) called *Energy in a Finite World*. (Specific references in what follows are to page numbers in the published report.) The implied accusation is that the modelling is biased to emphasize the potential role of the fast breeder reactor, but that only small changes in the parameters involved show this solution to be unnecessary. This requires a rebuttal.

Keepin and Wynne's big mistake is to have confused mathematical modelling of scenarios with the rest of the book. Our conclusions are not to be found in the chapters on mathematical modelling, which are not meant to anticipate the conclusions, which are themselves given at the end of the book (chapter 25).

The conclusion that matters there is that it could be done" (p. 771). The conclusion is not that it will, should or even can be done. The use of the conditional rather than the indicative verb refers implicitly to the overall assumptions, that the world will not experience major catastrophes, that, overall, it will behave rationally and cooperatively and that the gap between developed and developing countries will be closed, albeit slowly. In other words, we asserted that primary energy supply *could* meet future energy demand.

Whether the real world develops in this way is deliberately left open. Indeed, we conclude that "the reality of political, societal and institutional problems will probably make the situation more grim than has been described in our two scenarios" (p. 778). So the study is meant to serve for purposes of orientation and provision of perspective, not as a prediction of reality, as was recognized when the study was considered "sensible" in an editorial note³ in *Nature*.

The conclusion that "the demand for liquid fuels is a principal driving force of the energy problem" (p. 779) was a surprise not in the minds of the authors when the study was begun in 1973. They indeed expected that fast breeders would assume a major role, if not the major role, in the future; the study showed something different, a problem within the energy problem.

So much, the authors learned; they had come a long way, which led to fossil, not nuclear, fuels. If the study makes a case at all, it is the case for fossil fuels. On the global scale, even low quality fuels were recognized to be important, whence our concern for environmental issues and the issue of the cleanliness of fossil fuel usage (pp. 781, 804). Since then, the cleanliness of fossil fuel usage has indeed become a political issue (the SO₂, NO_x case). But in

the late 1970s, when the study was concluded, that was not in everybody's mind.

In contrast with these explicit conclusions, there is no such explicit conclusion about the fast breeder, which enters only indirectly. Nuclear power must fill a gap when, by 2030, a total of between 24 and 36 TW-years per year of primary energy must be provided in our scenarios (p. 787). If one wants to postpone the use of nuclear power, these sources are already stretched to their limits. After all, our study was not considering just the United States as were Keepin and Wynne, but the whole globe. Indeed, our study even supposed that the United States would take on the role of coal supplier for Western Europe and Japan.

But if nuclear power is to assume the role of providing 5–8 TW-yr yr⁻¹, it will be necessary to have a large number of breeders. They will follow the learning curve, so that their costs will decrease. This is a top/down approach and not in logical contradiction with a bottom/up approach which, in a different context, would allow that there would not be many breeders, so that learning would not take place.

This dichotomy between a top/down approach and a bottom/up approach points to what is inevitably lost when there is no substantial number of breeders. We might not be able to provide 3–4 kW per head of the world's population. Indeed, the study notes that "nuclear waste and proliferation issues could limit the build-up of nuclear energy over the next fifty years" (p. 779) and so is quite explicit on this issue.

Keepin and Wynne's underlying proposition is to do without nuclear power by not using energy, that is by energy conservation. In the countries of the Organization for Economic Cooperation and Development (OECD), this leads mostly to the substitution of energy by capital and to drastic changes of lifestyle. Our study concludes that "only radical changes of lifestyle could lead to very low energy demand" (p. 773); we also say that "our Low Scenario implies strong — but probably more realisable — energy conservational measures" (p. 774). We continue to have strong doubts about the so-called soft-energy paths, but it is possible that their advocates could arrive at a comprehensive and global energy study, such as *Energy in a Finite World*, dealing not just with the OECD countries or only the United States but with the world as a whole, including the developing countries. Such a study might change our minds.

So far in this rebuttal, we have referred only to chapter 25 of our study, which contains our conclusions. What then was the function of mathematical scenario writing in the study? Mathematical scenario writing

was but one tool among others. The study considered various strata with both qualitative and quantitative insights. The quantitative insights, mathematical modelling matching energy demand and supply in various parts of the world, had then to be synthesized with the insights derived from other levels of the study, elements in the formidable task of drawing conclusions.

Keepin and Wynne have thus not recognized the structure of our study. They separated a particular part of it and made it appear as if the conclusions were derived exclusively from that.

The more specific questions of mathematical modelling in general and linear programming in particular have been addressed in an earlier rebuttal⁴; reference is made to that for readers who are interested in these details.

This letter was received in the *Nature* office on 1 February 1985.

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Radiation risks

SIR—As a member of the team cooperating with the Medical Research Council in its study of mortality among people working for the UK Atomic Energy Authority, may I say how pleased my colleagues and I were to see in *Nature* (22 August, p. 666) a balanced and generally accurate resume of the papers recently published in the *British Medical Journal*. So much could certainly not be said for much of the national press.

There is, however, one inaccuracy that could lead to misunderstanding. The number of deaths from prostate cancer (28 not 38 as you report) within the authority is precisely the number that you would expect on the basis of national rates (see Table III of the paper) and is clearly not excessive. The excess you mention refers only to a subgroup of workers who had been monitored for tritium; six deaths occurred when the expectation was only 0.67. Whether or not this is fortuitous is not known, although further investigations are to be conducted. It should be noted, however, that the overall mortality of tritium workers is exceptionally good, being only 60 per cent of the nationally based expectation (see Table VII).

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Events at the surface of the cell

Cell division requires a simple external signal to be converted into a complex series of internal events. Attention to that process would help more than further oncogene sequences in understanding cancer.

AMONG speakers at last month's EMBO Symposium on Growth Factors, Receptors and Oncogenes (Heidelberg, 16–19 September) the most common currency was a figure along the lines of that shown here. The exact version differed according to the interests and beliefs of individual speakers. But all attested to the growing recognition that the hard facts of molecular biology have, sooner rather than later, to be integrated with cell physiology and even plain old biochemistry if they are not to be biologically sterile.

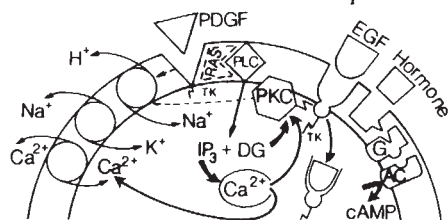
This is nowhere more so than in the case of oncogenes, where molecular information on gene and protein sequences has far outstripped understanding of how the proteins are involved in tumorigenesis. And exactly the same can be said of the genes from which oncogenes are derived (by mutation) and whose proteins are involved in normally regulated cell growth. Thus we know the sequences of many normal growth factors, a few of their cell-surface receptors and a number of oncogenes that are known or suspected to be mutants of growth factor or receptor genes, but much less about how the signal generated by a growth factor meeting its receptor on the exterior surface of a cell's membrane is converted into internal instructions that lead to cell division.

At least something is known, of the initial events. Thus the binding of some growth factors, including platelet-derived and epidermal growth factors (PDGF and EGF), to their receptors, activates a tyrosine kinase (TK) activity on the receptors' internal tail. Some ligand-receptor complexes, including EGF-receptor, are internalized. The PDGF-receptor complex, unlike the EGF-receptor complex, activates phospholipase C (PLC), which generates inositol trisphosphate (IP_3) and diacylglycerol (DG). IP_3 leads to the release of intracellularly stored calcium ions (Ca^{2+}); DG activates protein kinase C (PKC), which seems to trigger Na^+/H^+ exchange, accounting for the transient increase in pH that characterizes growth factor-stimulated cells. But some of these links are poorly understood and there are many others to be forged if the initially observed events in a stimulated cell are to be explained, let alone the end result of regulated or, in cancer, unregulated cell division.

One particular puzzle is where the protein products of the *ras* genes fit in. These proteins are located in the cell surface,

bind GTP and hydrolyse it. Certain mutations convert the genes into oncogenes, the protein products of which are much less good at hydrolysing GTP. With the collapse (see *Nature News and Views* 5 September, p.16) of the idea that *ras* proteins might be the GTP binding proteins (G) that convert a hormonal signal at the cell surface into the production of cyclic AMP by adenylate cyclase (AC), attention at Heidelberg was turned to the possibility that *ras* proteins (RAS) are the link between receptors, such as that for PDGF, and phospholipase C (PLC). But so far it is hopes rather than evidence that keep that idea afloat.

Whereas the rise in internal Ca^{2+} that rapidly follows PDGF stimulation of a cell seems to be a consequence of the release of internally stored Ca^{2+} , that following EGF stimulation is the result of an inward flux of extracellular Ca^{2+} , according to W. H. Moolenaar of the Hubrecht Laboratory in Utrecht. By generating monoclonal antibodies to the EGF receptor that



Initial events at the surface of a stimulated cell.

mimic EGF in terms of stimulating TK but not in raising internal Ca^{2+} , he has neatly dissociated the two responses. The EGF receptor was the focus of attention by speakers from several laboratories, in part because it provides a good example of receptor 'transmodulation'. In this example, the affinity of EGF receptor for EGF is decreased by the binding of PDGF to its own receptor. It seems that the mechanism hinges on PKC which, activated by PDGF binding, phosphorylates EGF receptor on a serine residue that lies just on the cytoplasmic side of the cell's membrane. (How that decreases the affinity of the extracellular binding domain of the receptor or, for that matter, how EGF binding activates the intracellular TK, are pressing questions.) A contrasting example of transmodulation is the binding of insulin to adipocytes. This increases the number of insulin-like growth factor receptors on the cell surface by decreasing their phosphorylation, which inhibits their internalization, suggested M.P. Czech of

the University of Massachusetts.

Whereas there is at least some understanding of the initial events mediated at or via the surface of a stimulated cell, next to nothing is known about the process by which later events are induced. For now, the emphasis is on finding out which genes, and in what order, are induced by stimulation. In that respect, a fascinating new turn of events, described by C.D. Stiles of the Dana-Farber Cancer Institute in Boston, is the increase in β -interferon and 2',5'-oligoadenylate synthetase gene transcripts after the stimulation of fibroblasts by PDGF, although considerably later in time than the previously documented induction of *myc* and *fos* (which are both implicated as oncogenes in other circumstances). Interferon and the synthetase are well known to be induced by viruses and are thought to mediate the antiviral response of cells, with the synthetase generating 2',5'-adenylate which then activates a ribonuclease that is supposed to have a specificity for viral messenger RNA. Stiles suggests that the specificity is broader and perhaps includes *myc*, *fos* and other messenger RNAs that must only be allowed a transient appearance in stimulated cells if their growth is to be normally regulated. In complementary experiments reported by Stiles, vesicular stomatitis virus or poly (I:C) induced *myc* and *fos* before interferon and the synthetase, and in much the same manner as PDGF.

In fully (serum) stimulated cells there are perhaps 100 induced genes according to D. Nathans of Johns Hopkins University. Either luck or brute force will be needed to identify the key members of such a large set. Key member or not, one of 100 genes is of considerable interest in that it encodes a new hormone, proliferin, which is related by sequence to prolactin. In mouse tissues, a proliferin-like protein is found only in the placenta, which contains messenger RNAs for two proliferin-like peptides.

With the emergence of new hormones and reports of new receptors (several laboratories have characterized a receptor that is closely related to the EGF receptor, but none has found its ligand), oncogenes (including one that contains a large segment of a tropomyosin gene) and inositol phosphates (notably a 1,3,4-isomer of the usual 1,4,5- IP_3), the likelihood is that any figure of the type shown is destined soon to become a palimpsest. **Peter Newmark**

Cometary science

ICE encounters Giacobini-Zinner

from Stanley W. Cowley

ON 11 September 1985 the NASA International Cometary Explorer (ICE) spacecraft made the first ever close fly-by of a comet, passing through the tail of comet Giacobini-Zinner 7,800 km from its icy nucleus. Two days later, the first results presented at Goddard Space Flight Center, Maryland, by the principal investigators of seven ICE experiments, revealed a rich and detailed data set.

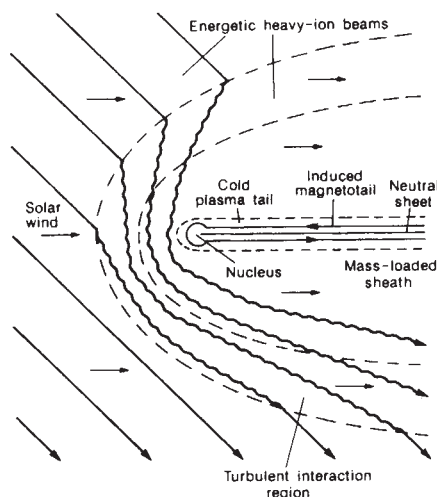
The results have already ended more than thirty years of speculation on the nature of solar wind-comet interactions by showing how the comet's atmosphere interacts with the solar wind, the supersonic proton-electron plasma that blows continuously outward from the Sun into the Solar System at speeds of about 400 km s⁻¹. In particular, it has been verified that the interaction produces copious fluxes of energetic heavy ions in the region surrounding the comet, resulting from solar UV ionization of the extended cometary atmosphere followed by ion pick-up by the solar wind flow. The wind flow becomes mass-loaded and slowed in the process; the long, filamentary ion tails of comets are formed by the capture of magnetic flux from the solar wind resulting from this mass-loading, as first theorized by H. Alfvén (*Tellus* 9, 92; 1957).

The ICE spacecraft was launched in August 1978 as ISEE-3 (International Sun-Earth Explorer-3). After a successful four-year programme to study particles and fields in the solar wind, it made the first detailed observations of the Earth's extended magnetic tail (see *Nature News and Views* 315, 281; 1985). A final lunar swing-by on 22 December redirected ISEE-3 towards Giacobini-Zinner. On successful completion of this manoeuvre ISEE-3 was renamed ICE.

Giacobini-Zinner is a periodic comet, independently discovered by M. Giacobini in 1900 and E. Zinner in 1913. The elliptical orbit has a period of 6.5 years, and lies between the orbits of Earth and Jupiter. At the time of the ICE encounter the comet was six days past its closest approach to the Sun and was crossing the ecliptic plane at an angle of about 30°. Fortuitously, the component of the comet's speed in the plane of the ecliptic nearly matched that of ICE (30 km s⁻¹), so that Giacobini-Zinner made a simple north to south pass across the spacecraft at a sedate relative speed of 21 km s⁻¹. ICE was targeted so that the centre of the comet's tail should have passed across it, 10,000 km from the nucleus. But due to a hiccup in the comet's orbital parameters, the nucleus was only 7,800 km from the spacecraft, causing considerable concern that cometary dust would inactivate the

spacecraft's solar cells. ICE survived the encounter with no measureable degradation in performance, although plasma bursts produced by the impact of micrometre-sized particles on the spacecraft body may account for the wave bursts detected at a rate of about one every second during the hour of closest approach, as reported by F.L. Scarf (TRW Systems, California).

Phenomena associated with the interaction of solar wind with the cometary atmosphere were observed over a much longer interval. The atmosphere results from sublimation of the icy surface material of the nucleus (itself about a kilometre in diameter), and is believed to expand outwards, essentially unrestrained by gravity, at about 1 km s⁻¹. As the gases (mainly water vapour) expand, they undergo complex photochemical reactions with sunlight, leading to breakup and ionization of the initial molecules. Time scales for the latter processes are of the order of 10⁵–10⁶ s, so that with about 1 km s⁻¹ expansion speeds, these phenomena are expected to extend 10⁵–10⁶ km from the nucleus. Upon ionization the ions are picked up by the solar wind flow (~400 km s⁻¹) and accelerated to kinetic energies proportional to their mass, typically a few tens of keV for O⁺ or OH⁺. As reported by R.J. Hynds (Imperial College, London), pickup ions with these energies were detected by the energetic ion experiment run by Imperial College/SSD-ESA/Space Research Laboratory Utrecht more than one day before the spacecraft's closest approach, when it was still 2 mil-



Giacobini-Zinner's interaction with the solar wind. The cold plasma tail is about 25,000 km across. The solid icy nucleus (not to scale) is probably only a few km across. Short arrows indicate the direction of plasma flow away from the Sun. Solid lines indicate magnetic field lines lying approximately in the ecliptic plane.

lion km from the comet. These observations constituted the first evidence of the approaching comet in the ICE data. Although bursty in nature, peak fluxes generally increased as the comet was approached, reaching values higher than any seen in the previous seven years of the experiment. Confirmation that these ions were low charge-state heavy ions was provided by the energetic ion experiment of the Max-Planck Institute, Garching and the University of Maryland, as reported by D. Hovestadt (MPI, Garching).

Although the production of copious fluxes of heavy ions by solar wind pick-up is confirmed to be a unique feature of the solar wind-comet interaction, the energy and momentum input to energetic ions close to the comet may be expected to become so large that substantial slowing of the flow occurs (the flow becomes 'mass loaded'). The magnetic field which is frozen into the highly conducting plasma should then become distorted and 'draped' around the cometary obstacle (see figure). This was broadly confirmed by the ICE thermal-electron and magnetic-field experiments, described by S.J. Bame (Los Alamos National Laboratory) and E.J. Smith (Jet Propulsion Laboratory), respectively.

An overview based largely on these results is shown in the figure. No clear shock was observed in the magnetometer data in the outer part of the mass-loaded region, but a broad, turbulent interaction region was found 70,000–120,000 km from the comet's nucleus. Intense plasma waves were detected in this region. Inside this broad turbulent layer a less disturbed 'sheath' region of slowed flows was detected before ICE crossed the cold plasma tail of the comet for a 20 min interval centred on closest approach. Thus, the tail is 25,000 km across, and is characterized by draped magnetic fields and cold electron densities of about 100 cm⁻³. Within this region is an induced bipolar magnetotail with embedded neutral sheet, which presumably results from cometary capture of solar wind magnetic flux by mass-loading. Electron densities in the heart of this tail, deduced from measurements made by the radio-wave experiment, were reported by J.-L. Steinberg (Paris Observatory, Meudon) to exceed 600 cm⁻³ in a ~1,000-km thick layer, with energies ~3 eV. The predominant ion in this region is from the 'water group' though some CO⁺ may also be present, according to K.W. Ogilvie (Goddard Space Flight Center).

Many more months of study will be required before the full implications are realized. But it is already clear that a quantum leap has taken place in knowledge and understanding of the atmospheres of comets and their interactions with the solar wind. □

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Virus structure

First comparison of two animal viruses in three dimensions

from Stephen C. Harrison

THE full three-dimensional structures of two small, positive-stranded RNA animal viruses, reported recently in *Nature*¹ and in *Science*², are dramatic crystallographic achievements as well as major contributions to structural virology. The technical significance of the human rhinovirus (HRV14) structure described by Rossmann and colleagues¹ has already been detailed in these columns³. With the publication of the poliovirus structure by Hogle, Chow and Filmen², the structure of these two related viruses can now be compared. It is clear that they have many remarkable details in common, a number of which they also share with the RNA plant viruses. Moreover, the structures have important implications for assembly, antigenicity, receptor binding and uncoating.

The so-called picornaviruses, of which poliovirus and rhinovirus are examples, are built from 60 copies of each of four viral proteins, known as VP1–4, and from a single genomic RNA with a small protein (VPg) at its 5' terminus⁴. All the viral proteins are synthesized as part of a polyprotein precursor cleaved as shown in Fig. 1a. The cleavages generating VP0 (= VP4 + VP2), VP3, and VP1 are carried out by a virus-specific protease. Sixty copies of VP0, VP1 and VP3 can form an RNA-free shell, known as a procapsid and believed to be a precursor to the virus particle⁴. The cleavage of VP0 to VP4+VP2, required for RNA incorporation, may be autocatalytic or it may be facilitated by some other part of the capsid structure. The ma-

ture virion appears to contain one or two uncleaved VP0 chains.

How do these proteins fold and how do they form a virus particle? The poliovirus and rhinovirus structures show that the folded polypeptide chains of VP1, 2 and 3 all have very similar core structures — β rolls that are strikingly similar to the S domains of tomato bushy stunt virus (TBSV) and southern bean mosaic virus^{5–7} (see Fig. 1b). These compactly folded cores have long N-terminal extensions, shorter C-terminal extensions, and signifi-

cant insertions at the positions of one or more loops between β strands. VP4 is, in effect, part of the N-terminal extension of VP2. The proteins are packed in the icosahedrally symmetrical particle, as shown in Fig. 1c, with the N-terminal extensions (including VP4) toward the inside of the particle. In poliovirus and HRV14, the character and length of the extensions and insertions are very similar, but comparison with other picornaviruses suggests some variability in these elaborations, probably significant for antigenicity.

No features attributable to RNA are seen in either of the new electron density maps. The crystallographic methods used would only have seen RNA segments bound in identical ways to all 60 protomers. Thus, just as in the plant viruses^{8–10}, there is no fixed way in which RNA interacts with ordered parts of the protein.

The disposition of N-terminal arms in

these structures is remarkable. The arm of VP1 folds into a large loop lying against the inner surface of VP3. The arm of VP3 extends beneath VP1 from its N-terminus near the 5-fold axis of the particle, where it interacts with four other symmetrically related structures in a five-stranded β structure that Hogle *et al.*² describe as a twisted tube. The N-terminus of VP4 lies near the twisted tube, so that five VP4 segments contribute to the 5-fold structure. The remaining part of VP4 extends toward the particle's 3-fold symmetry axis, and its C-terminus lies near the N-terminus of VP2, from which it is cleaved. This description, shown in the diagram in Fig. 1c, implies that the arms can fold only as the subunits associate — that is, the important tertiary structure of the N-terminal extensions cannot be realized without the quaternary organization of the capsid.

What are the implications of this pattern of organization for viral assembly? Steps thought to occur in the virion formation pathway include the initial

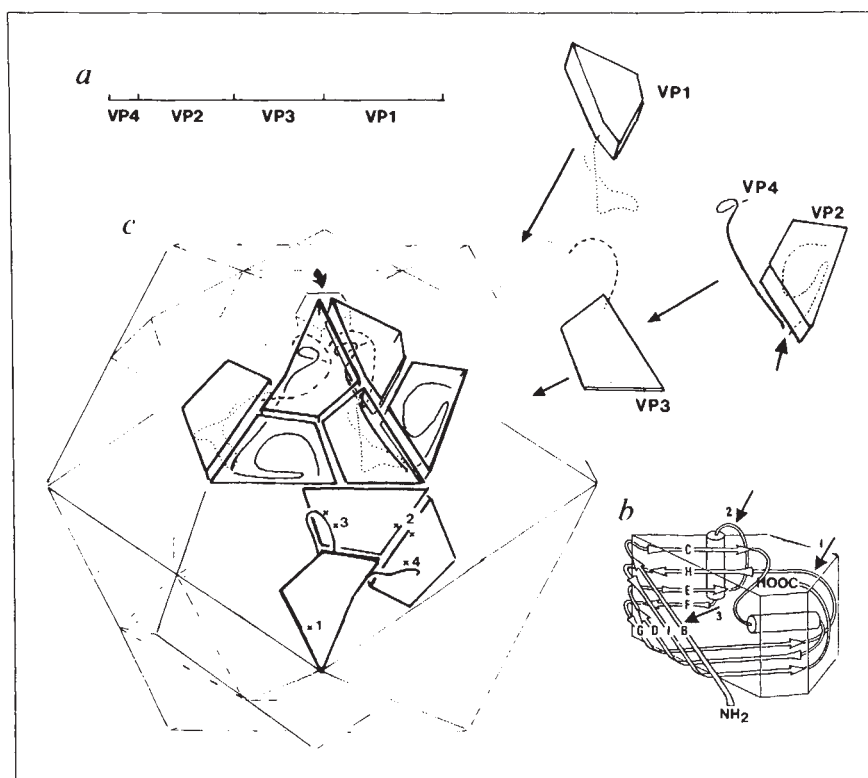


Fig. 1. a, Order and approximate relative sizes of VP1–4 in the polyprotein from which they are cleaved. b, Folded structure of the polypeptide chain in the 'core' of VP1, VP2 and VP3 (adapted from Hogle *et al.*, ref. 2). Such a domain, based on two β sheets, is sometimes termed a 'swiss roll' or 'jelly roll'. The arrows labelled 1, 2 and 3 indicate the positions of significant insertions in VP1, VP2 and VP3 respectively, both in poliovirus and HRV14. Since the upper part of the diagram corresponds to the outside of the virion, all the insertions, as well as the C-terminus, can be seen to project outwards. c, Packing of VP1–4 in the capsids of HRV14 and poliovirus. Three protomers (1 copy each of VP1–4) are shown, with the elements of the upper right-hand protomer exploded from the icosahedral particle. The full capsid contains 60 protomers, symmetrically covering the icosahedral shell (see Fig. 2b in ref. 1). Here special attention is given to the N-terminal arms of VP1–3 and to VP4 (drawn as for poliovirus; HRV14 seems essentially similar). The arm of VP1 is shown dotted, that of VP3 dashed and the arm of VP2 and all of VP4 solid. Note the way the arm of VP3 loops under VP1 and interacts with four other VP3 arms in a twisted tube around the 5-fold axis (indicated by heavy curved arrow and outlines of a pentagonal prism). A loop near the N-terminus of VP4 also 'backs up' this twisted tube structure. The point at which VP4 is cleaved from VP2 is shown by an arrow on the exploded VP2 subunit. Clearly VP4 is part of the N-terminal extension of VP2. In the protomer in the lower half of the figure, positions are indicated of four clusters of residues (numerals 1–4), identified by Hogle *et al.*² as principal neutralization sites in poliovirus. Similar sites have been identified in HRV14 (refs 1, 13). The insertion and C-terminal extension of VP1 are also shown, as residues in them contribute to clusters 3 and 4 respectively. Note how they enhance the overall contribution of VP1 to the viral surface.

Rodney Robert Porter (1917–1985)

RODNEY PORTER, best known for his landmark elucidation of the structure of antibody molecules, was killed on September 6 in an automobile accident that tragically ended the life of an extraordinarily respected and beloved scientist a month before his 68th birthday. Only weeks earlier, a special meeting had been held in Oxford to mark his retirement from the Whitley Chair of Biochemistry, though he was to have continued as director of the Medical Research Council's Immunochemistry Unit for another four years. The subjects discussed in the meeting by Porter's former students and associates, along with present members of the Unit, were those that had been of particular interest to Porter in recent years, especially the structure and genetics of complement components and their cellular receptors.

Porter's interest in the immune system was aroused in 1946 when, as a graduate student under the supervision of Fred Sanger in the Cambridge University Biochemistry Department, he read the monograph that inspired a generation of immunologists, Landsteiner's classic, *The Specificity of Serological Reactions*, which delineated the remarkable range of antibody specificity. Since most antibodies are indistinguishable in overall molecular properties and even in antigenicity, the extraordinary diversity in combining specificity seemed paradoxical. As Porter stated in his Nobel lecture (*Science* 180, 713; 1973) in characteristically simple and clear terms; "This combination of an apparently infinite range of antibody combining specificity associated with what appeared to be a nearly homogeneous group of proteins astonished me and indeed still does". It was to the resolution of this "antibody paradox" that Porter's research was directed during the first phase of his career.

By the 1950s, methods had been developed for determining the amino-acid sequence of proteins, but these techniques were not then applicable to molecules the size of antibodies. Porter recognized that it

would be necessary to fragment the antibody molecule to find a smaller piece that retained the activity. In experiments carried out initially in Cambridge and subsequently refined in the late 1950s at the National Institute for Medical Research at Mill Hill, he found that the enzyme papain cleaved rabbit IgG antibodies into three pieces of similar size: two identical Fab fragments, each of which carried a combining site and with it the inherent variability of the parent molecule, and an Fc piece which — most surprisingly — crystallized. "This last observation suggesting that a protein that itself would never crystallize could give a fragment that presumably was more homogeneous and hence able to crystallize, was quite unexpected and indeed was unacceptable", wrote Porter in his Nobel lecture. Later, Porter took great pleasure in telling how for several months he threw the Fc crystals down the sink in the belief that they were crystals of cystine, derived from the cysteine that had been used to activate the papain. Finding common and variable structures within a single protein molecule provided the key to the solution of the antibody paradox.

Also in the late 1950s, Gerald Edelman at the Rockefeller Institute demonstrated that antibodies are multi-chain proteins. The remaining problem was to relate the papain pieces to the chains, which necessitated isolation of the chains in soluble form. This was accomplished in the early 1960s in Porter's laboratory, then located at St Mary's Hospital Medical School in London. Antigenic analysis of the separated heavy and light chains with antisera to the Fab and Fc fragments played a crucial role in providing evidence for the four-chain model, which was proposed by Porter in 1962. For their contributions toward establishing the basic structure of the antibody molecule, Porter and Edelman were jointly awarded the 1972 Nobel Prize in Physiology or Medicine.

After moving to Oxford in 1967, Porter

and his colleagues extended their investigations of immune phenomena from the antibody molecule to complement, effectively applying the methodology of protein chemistry and DNA technology to unravel some of the molecular complexities of this system. Their studies focused on the structure and activation of the early components. An intriguing result was the determination of the unusual structure of the C1q subcomponent that binds to antibodies — its six globular heads and collagen-like stem resemble a bunch of tulips.

Recently, Porter's group established the order of the complement genes in the major histocompatibility gene complex (MHC). One of the components encoded by these genes, the highly polymorphic C4, occupies a key position in the complement cascade, binding covalently to antibodies and cell-surface antigens and non-covalently to other complement components and control proteins. Porter speculated that the polymorphism of C4, by affecting the level of complement activation, might account for the association of MHC with certain disease states. Porter enjoyed working at the bench and was planning to begin his 'retirement' by attempting to crystallize C4 proteins.

All who were acquainted with Rodney Porter agree that he was a highly original individual who profoundly affected everyone who crossed his path. In his science, as in his life, he focused on the central issues. Whether investigating the structure of a complex protein or tramping across the countryside he followed a purposeful, though often unmarked, route. Byways did not distract him; with gaze forward, he noticed and remembered everything he passed while others, looking in all directions, missed half the landscape. Although his famous walking speed may have decreased somewhat in recent years, the rigor of his scientific thinking and the vigour of his leadership were undiminished. In Porter there was an exceptional integration of man and scientist and he was as much loved for his unique character as he was respected for the accomplishments that sprang from it. L.A. Steiner

processing cleavages to form VP0, VP1 and VP3, association of these units to form a 6S protomer, and association of 6S species into pentameric 14S structures. The VP0/VP1/VP3 assembly unit in these viruses is clearly defined by the interactions of the N-terminal arms just described, as well as by the dispositions of C-terminal extensions from VP1 and VP3 (see Fig. 1c). The significance of the 14S species (a pentamer of these protomeric assembly units) is also clear from the convergence of five VP3 N-termini in the twisted tube and their association with segments of VP4 (Fig. 1c). The phenomenon of regulating assembly by interaction of extended arms was first discovered in the plant viruses^{5,6}. The assembling units are not just rigid, preformed 'bricks'; they

have extensions that reach across adjacent units to form second- and third-nearest neighbour interactions.

Another conclusion about assembly that can be drawn from the HRV14 and poliovirus structures is pointed out in both papers^{1,2}: cleavage of VP0, VP1 and VP3 from each other is probably an early event, preceding formation of the 6S protomer, since the C- and N-termini generated by the cleavages are not at all near each other in the virion. Indeed, N-termini are on the inside of the particle and C-termini are on the outside.

By contrast, the proximity of the C-terminus of VP4 and the N-terminus of VP2 is consistent with the later occurrence of the corresponding cleavage. The position and character of this junction gives no

obvious clue, however, to the observed linkage between VP0 cleavage and RNA incorporation. Polio- and rhinovirus proteins, unlike many plant-virus coat proteins and unlike the core subunits of alphaviruses, do not have positively charged 'R-domains' at the extremities of their N-terminal extensions. The mechanism of RNA condensation in these structures is therefore not yet evident. It is probably appropriate to consider again whether RNA is inserted into a preformed capsid, as suggested by previous *in vivo* kinetic data on poliovirus, or whether RNA is condensed by association with 14S pentamers as they assemble into a shell.

The implications of these structures for studies of viral antigenicity are no less striking. Viral epitopes can be assigned by

various methods but probably the most specific involves isolation and characterization of 'monoclonal release mutants' — viral mutants selected for resistance to a neutralizing monoclonal antibody. Correlation of such mutations with three-dimensional structure of a viral protein was shown to be useful by Wiley *et al.*¹⁰, who found that in epitopes thus identified in influenza virus, haemagglutinin clustered on the surface of the subunit, often in protruding loops. Hogle *et al.*² comment that sites located in this way in poliovirus^{11,12} appear to cluster into three or four patches on the surface (see Fig. 1c) and a similar but not identical set of patches in HRV14 are described by Rossmann *et al.*¹ from the work of Sherry and Rueckert¹³. All the mutation sites occur in exposed loops, which are fully accessible to antibody binding. These loops include all the protruding insertions in the three principal poliovirus subunits and two of the three in HRV14. The polio VP1 C-terminus, draped over VP3, is also implicated. Residues in such loops can probably be more variable in sequence than residues in the core.

The way in which the insertions and C-terminal extensions decorate much of the viral exterior suggests a mechanism of antigenic disguise, in which potentially variable structures cover much of the accessible antigenic surface. Similar suggestions have been made for influenza virus haemagglutinin, where the conserved residues essential for receptor (sialic acid) binding are in a deep pocket, inaccessible to antibodies¹⁴, and where acquisition of a glycosylation site in one strain has led to the covering of a protein epitope by the new oligosaccharide¹⁵.

The unanticipated similarity of the cores of VP1, VP2 and VP3 to each other and to the S-domains of plant-virus coat proteins emphasizes how alike small positive-stranded RNA viruses are in both plant and animal hosts. Such a relationship was already evident from the homology of viral RNA-dependent RNA polymerases, pointed out by several groups¹⁶⁻¹⁸. The implications for our understanding of viral evolution cannot yet be adequately assessed. A possibility, with important consequences for virology, is that animal and plant viruses share a fairly recent history, perhaps through the mediation of insect hosts.

An important factor for tissues tropism and pathogenesis of a virus is its binding to receptors. Picornavirus receptors have been partly characterized but not yet purified, and different members of the family appear to have different receptors⁴. The shape and packing of VP1 gives rise to a depression on the surfaces of HRV14 and of poliovirus, which Rossmann *et al.*¹ suggest may be the site of receptor interaction. VP1 has been implicated in receptor binding in foot-and-mouth disease virus¹⁹.

One consequence of binding to a cell is that these viruses undergo a conforma-

tional change that leads to release of VP4 (ref. 4). Since VP4 is an internal protein, and since the core domains are rather tightly packed, the shell must expand or breathe in some manner for VP4 to escape. The expansion of plant viruses such as TBSV provides a plausible model for such a process²⁰. This transformation is a cooperative rearrangement of subunit packing in the shell, conserving many of the interactions in the native particle while a few others are altered or ruptured.

It has been suggested²¹ that neutralization of poliovirus involves cross-linking of a few subunits by divalent antibody molecules, which would indeed inhibit a cooperative structural transition. Studying altered forms of the picornavirus particles may thus provide significant insight into initial events in uncoating. Understanding of subsequent events will require information not yet available from cell biological experiments on the form in which RNA enters the cytoplasm. Is it still encapsidated (for example, by VP1-3), or is uncoating coupled to membrane traversal? Either possibility leads to structural puzzles.

It should be evident from this summary that X-ray crystallography has just begun to contribute to our understanding of virus assembly and viral pathogenesis. The two structures just completed show that the technology and methodology — developed in work on plant virus structures^{6-8,22} and first applied to problems of human viral infection in work on influenza virus haemagglutinin²³ — are firmly in place. Indeed, the HRV14 and polio virus stu-

dies make an interesting contrast in this respect, for Rossmann's group has used synchrotron data collection and a super-computer, whereas Hogle's group has relied on a laboratory X-ray generator and a small VAX computer. Methods aside, what is evident from the parallel features of these two structures is that elucidation of the regulated assembly, of the complex antigenicity, and of the incompletely characterized binding and uncoating of these viruses can now proceed from clear three-dimensional pictures. □

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Geology

Rise and fall of periodicity

from David M. Raup

THE question of whether major extinctions in the history of life have a clocklike spacing has been hotly debated. Some good syntheses of palaeontological data have resulted, and the debate has produced a general search for other periodic signals in the Earth's history. One possibility is periodicity in the geological record of the reversals of the Earth's magnetic field — though the consensus has been (and perhaps still is) that the fine structure of the magnetic record is purely stochastic. A danger with all such analyses is that they tend to yield "an answer", which may hide real problems with the data set and statistical methods used, as is well illustrated by the recent history of this subject.

In 1983, Negi and Tirwari argued for a 32-Myr stationary periodicity in the magnetic field and Mazaud *et al.*² claimed a 15-Myr periodicity. Earlier this year, from my own analysis³, I recognized a 30-Myr signal in phase with, and possibly a harmonic of, the 15-Myr signal. The 15-Myr analysis was debated in *Nature*^{4,5} and

now, on page 409 of this issue⁶, Lutz challenges my case for a 30-Myr periodicity. He has shown by an elegant experiment that the 30-Myr signal is predictably sensitive to the length of the time series: when the record is truncated by progressively eliminating the most recent events, the spectrum changes, showing that the 30-Myr peak is an accident of record length. Lutz is correct. And I apologize to the readers of my earlier paper.

While the implications of Lutz's study for the other claims of periodicity in magnetic reversals are not yet clear, since those studies used different statistical techniques, they will now have to be examined very carefully. The new result also has implications for the periodicities in biological extinction⁷⁻⁹ and impact cratering¹⁰ that have been claimed. Two of the three studies of extinction used essentially the same statistical techniques that I used with the magnetic data^{3,9} but, as Lutz points out, the extinction and magnetic data are different. I am happy to report

that Lutz's truncation procedure has been applied to the analysis of the extinction data with no effect on its results.

Let me discuss more generally what a claim of periodicity in the history of the Earth, or its biology, really means. In almost any statistical analysis of the timing of events, the first job is to see if a null hypothesis of randomness can be rejected. This is convenient in the present case because random spacing in time, which could result from multiple, independent causes, happens also to be the chief conventional wisdom in geology and palaeontology. But there is an unfortunate misconception about random patterns that makes the research difficult: most people tend to equate random spacing with uniform spacing. For example, it is easy to think that a 30-year flood is due every 30 years and that we are safe for a considerable time after each such flood. Nothing could be further from the truth. Points randomly placed on a time line show a Poisson distribution and are almost always clustered in a counter-intuitive pattern, with long gaps between clusters. The statistical testing of the extinction record was started because the major events of the past 250 Myr looked much more evenly spaced than expected. The testing led to rejection of the random hypothesis — although this rejection is naturally only as good as the statistical methodology used.

Once the null hypothesis of randomness is rejected, a search for any particular non-random signals can begin. There is a special difficulty here, because of the nearly infinite and uncountable number of possible non-random signals that could describe the real sequence. Only a few of these can be tested in practice, and these are limited to the simplest models. When a claim is made that a simple periodicity, such as 26 or 30 Myr, fits geological or palaeontological data, all this really means is that the fit is better than for other signals that have been tried. One can rarely attach a confidence level in

a maximum likelihood sense to the claim, except in the context of the original rejection of the null hypothesis of randomness.

How important is the accuracy of the data that go into the analysis? Can rejection of the hypothesis of randomness be challenged if the dating or identification of events contains substantial uncertainty? The answer is generally no and this is again counter-intuitive. Suppose, for example, that we have a perfect periodic signal. Rejection of the random hypothesis in such a case would be straightforward and emphatic. Now suppose we degrade the data by adding random noise, say by moving points forward or back in time by randomly chosen amounts. This will change the sequence away from periodicity and towards randomness. Thus, the inclusion of uncertain data in the time series only makes tests of the null hypothesis more conservative: the worse the data, the less likely one is to be able to establish a non-random pattern. It is probably more prudent to include questionable data than to risk unconscious bias by removing them.

In my view, the claimed periodicities in the Earth's history should be seen as hypotheses for testing. Each claim is only as good as its ability to predict other correlated phenomena, be they companion stars or tectonic cycles, and the jury is still out on this confirmation. □

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Gene transcription

A pulling out of fingers

from Tariq Enver

AN EXCITING new structural clue about the mechanism of polymerase III transcription and the regulation of 5S RNA genes has recently been found¹. Two 5S RNA multigene families are found in *Xenopus*: the 'somatic-type' and the 'oocyte-type', which produce short, identically sized transcripts differing by only 6 nucleotides in sequence. The somatic-type genes are present at ~400 copies per haploid genome, being transcribed in both somatic cells and oocytes. The oocyte-type genes, which are present at ~20,000 copies per haploid genome, are only transcriptionally active in oocytes. 5S RNA

genes therefore provide a prime model system for the developmental control of eukaryotic gene expression (see refs 2,3 for review).

Both gene types are transcribed by RNA polymerase III and like all eukaryotic class III genes contain an internal control region (ICR)⁴, which directs upstream initiation by the enzyme. Polymerase III, however, is only able to recognize the ICR in the context of a transcription complex involving at least three other factors^{5,6}. One of these, a protein of relative molecular mass 40,000 (40K) called TFIIIA, constitutes up to 15 per cent of total oocyte

protein, interacts specifically with the ICR of 5S genes and is not required for the synthesis of any other polymerase III transcript. The binding of TFIIIA to the ICR is followed by the sequential binding of factors TFIIIC and TFIIIB. It is to this complex that RNA polymerase III binds⁸. As well as binding 5S DNA, TFIIIA is associated with 5S RNA in a 7S ribonucleoprotein particle that is the storage form of 5S RNA during oogenesis (see ref. 3). The ability to bind the transcription factor of its own gene suggests an attractive autoregulation mechanism for 5S RNA synthesis, based on feedback inhibition. The striking similarities between the 5S RNA-TFIIIA interaction and the 5S RNA-ribosomal protein interaction suggest that TFIIIA may be an evolutionary descendant of the ribosomal protein⁹.

TFIIIA is the first eukaryotic transcription factor to have been sequenced¹⁰; analysis of the sequence information reveals a segment in the TFIIIA coding region that contains significant sequence homology with the 5S ICR. One possibility this raises is that TFIIIA can interact with its own gene and messenger RNA in much the way that it does in the 5S system¹¹.

Although these models suggest mechanisms for the overall regulation of 5S RNA genes, they do not explain why oocyte-type genes are active in oocytes and repressed in somatic cells, whereas somatic-type genes function in both cell types. *In vitro* transcription experiments using cloned somatic and oocyte 5S templates in crude extracts have failed to reflect the transcriptional activity displayed by the two gene types in somatic cells¹¹. However, *in vitro* transcription experiments using purified chromatin templates have been successful in this regard. Further analysis of these templates reveal that the somatic-type genes are packaged in chromatin as active 'stable' transcription complexes, requiring only the addition of polymerase III for transcriptional activity. The repressed oocyte-type genes are not complexed with transcription factors and are prevented from recruiting these factors by a structure dependent on histone H1 (refs 11,12).

How then does the asymmetrical distribution of active and repressed stable complexes arise? The following model has been proposed¹¹: early in oogenesis in conditions of TFIIIA excess, large amounts of 5S RNA are synthesized as a result of the activity of both 5S RNA gene types. As 5S RNA accumulates, TFIIIA is recruited into 7S ribonucleoprotein storage particles. The cellular concentration of TFIIIA decreases during late oogenesis and on through early embryogenesis. This decline in TFIIIA concentration is accompanied by the full repression of the oocyte-type genes by the end of gastrulation. In the face of limiting amounts of TFIIIA, the fourfold stronger binding affinity of TFIIIA to the ICR of the somatic gene, which differs by two

base pairs from the ICR of the oocyte-type gene, could be sufficient to account for the observed asymmetrical distribution of stable active complexes. Generalized repression of those genes that have failed to recruit transcription factors occurs through the binding of H1.

As this stable complex is formed at a region internal to the transcriptional start of the gene, how is it able to withstand the continued cycling of RNA polymerase through it? Miller *et al.*¹ provide an elegant answer to this question. They show that the 7S ribonucleoprotein contains between 7 and 11 zinc atoms. Proteolytic digestion of the 40K TFIIIA protein yields the characteristic 30K and then 20K products used by Smith *et al.*¹³ for footprinting analyses; extended proteolysis yields a progression of intermediates spaced at 3K intervals, suggesting a periodic arrangement of small compact protein domains 3K in size. Computer analysis of the amino-acid sequence reveals nine copies of an imperfectly repeated motif of 30 amino acids, extending from residues 13–276 of the protein. Combining all this information, Miller *et al.* suggest that TFIIIA is composed of nine flexibly linked, small, compact, looplike domains, each enclosing a central zinc atom at its base by virtue of the two invariant pairs of cysteines and histidines (see figure). These looplike regions or 'fingers' are the proposed DNA binding regions. Differences in the amino-acid sequences at the 'fingertips' could reflect the lack of obvious nucleotide sequence repetition in the ICR. The remaining 70-residue stretch of TFIIIA has no homology with the repetitive zone, suggesting that it corresponds to the 10K domain required for efficient transcription but not for binding to the ICR.

This structure for TFIIIA is able to explain how the 5S transcription complex can remain stable despite the repeated passage of polymerase III. TFIIIA could disengage fingers bound near the process-

ing polymerase while remaining bound by its other fingers.

Sakonju and Brown have suggested that TFIIIA makes contact primarily with only one strand of the DNA helix¹⁴. If each finger interacts with one half turn of the helix as suggested, then the protein must spiral around the helix following the strand. Alternatively if the protein lies on one side of the helix, alternate fingers would have to interdigitate in different planes. Flexible hinging between the domains would make this possible.

Both the strand specificity and multiple contact binding of TFIIIA are reminiscent of another eukaryotic transcription factor, Sp1 (ref. 15), which binds to several short GC boxes in the early promoter region of simian virus 40 (SV40) and a related monkey cellular promoter. Some of the SV40 early unit transcriptional initiations occur within and upstream of the Sp1

binding site. Sp1 may therefore face some of the same problems as TFIIIA. Could, then, the use of multiple small contacts be a common feature of eukaryotic genetic regulatory proteins? Certainly the notion of amplifying an ancestral small domain would make good evolutionary sense. □

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Astrophysics

Lithium probes the Universe

from David N. Schramm

TWO DECADES ago lithium was considered an obscure light element, bypassed by mainline nucleosynthetic processes in stars, and thought to be synthesized with the other light nuclei in some mysterious proto-solar process (the 'x-process') — now it has become one of the confirming cornerstones of the big bang itself and one of the best probes of stellar evolution.

This transformation in the role of Li has been not so much a revolution as an evolution: each new result has appeared small but the accumulated result has been dramatic. Recently added to this 20 year history are two incremental but significant new Li results: those of Catherine Pilachowski² (Kitt Peak National Observatory) on lithium abundances in a moderately old star cluster, and those of V. Viola and his colleagues³ (University of Indiana) in a paper about nuclear cross sections for Li, Be and B. Both represent parts of far larger programmes at these two institutions on very different but tangentially related aspects of Li in astrophysics; they also complement independent major efforts to determine Li abundance at Paris and Chicago, and other cross-section measuring efforts at Michigan and California.

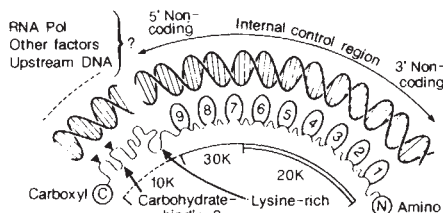
To put this new work in perspective let us remember that Li has two stable isotopes ⁷Li and ⁶Li, both of which are nuclearly fragile in that they are easily 'burned' away when exposed to protons at temperatures of a few million degrees, as encountered in stellar interiors. In addition, mainline nucleosynthesis occurs via the triple-alpha process which jumps from He to C and O, skipping over Li, Be, and B. As a result, these elements have abundances that are many orders of magnitude

below both He and C.

These meager but non-zero abundances have been intriguing; for although initially thought to be made along with deuterium by some proto-star process, it was subsequently shown by Hubert Reeves and his collaborators in Paris that proto-solar processes were not energetic enough to do the job of making any of the light nuclei, including D. Subsequent work by Reeves, Fowler, and Hoyle⁴ showed that Li, Be and B abundances could be roughly understood by spallation processes resulting from cosmic rays hitting interstellar material. (In fact, cosmic ray abundances of Li, Be and B are comparable to their neighbours.) Such spallation processes, however, fail to produce sufficient D and produce ⁷Li/⁶Li ≈ 2, whereas the observed meteoritic value is ≈ 12; thus ⁷Li must be supplemented. (The problem with the ¹⁰B/¹¹B isotopic abundance ratio of ≈ 4 versus the spallation cross-section ratio of 2.5 is still not unambiguously resolved.) Fortunately D and ⁷Li happen to be made in the big bang in just the right abundances⁵ to do the necessary supplementation if the density of baryons is ≈ 10 per cent of the critical value. These arguments have been strengthened considerably as spallation cross-sections have been determined at more energies with greater frequency.

This is where Viola's work fits. In particular, it has been shown⁶ from the cross-section that Li is overproduced in almost any process that can make D. These constraints are strengthened by Viola's new work on ⁴He + ⁴He → ⁷Li + ¹H, which support the cosmological role of D and ⁷Li.

The cosmological use of Li was further enhanced when the Spites⁷ found that old



An interpretation of the structural features of the protein TFIIIA and its interactions with DNA. The DNA is drawn curved, as if resting on a long beaded surface of the protein. The internal control region of the 5S RNA gene (bases 40–100) is drawn as six turns of DNA. The amino end is followed by nine repeats (residues 1–276) contacting the control region. The repeats may be extended DNA-binding fingers linked by flexible joints, each with a zinc-ion centre. The relationship between the grooves of the DNA and the positions of the protein is unknown. The drawing must not be taken literally but each unit binds to about a half-turn of DNA (modified from ref. 1).

Population II stars had Li on their surface, which did not appear to be depleted by stellar processing and gave a good fit to the Li abundance predicted by big bang nucleosynthesis from consistency with He and D. To this ^7Li from the big bang must be added ^7Li and ^6Li as well as Be and B from cosmic-ray spallation.

Looking at the surfaces of stars of different ages to trace the evolution of Li in the Galaxy is not easy: since Li is destroyed inside stars, any star with a deep enough convective surface or any star which went through a deep convective phase will burn its Li. For example, our Sun, a Population I star, severely depleted its surface Li, indicating either that the Sun's current convection zone goes deep enough that it is sufficiently hot to burn Li, or that during the proto-solar convective phase, the Sun was hot enough to deplete Li. It should be noted that standard solar models do not predict this solar Li depletion⁶; thus convective overshoot or pre-main sequence burning is required even for the Sun. The fact that some of the Spites' near solar-mass Population II stars did not burn their Li tells us that the depth of convective zones may change with composition.

Pilachowski's observation of Li in a moderately old (1.5×10^9 yr) star cluster, NGC 7789, being at a level comparable to the current interstellar Li abundance (big bang plus cosmic ray production) tells us that stars in this cluster did not deplete their Li either. To put this in perspective, young clusters, such as the Hyades, do show Li depletion in their solar mass and

lighter stars; lower-mass stars have deeper convective zones so they would deplete their Li even more. Thus an older cluster has had time to deplete. Field stars of the mass that are seen in NGC 7789 also show lower Li. Pilachowski argues that this non-depletion of Li may be due to some difference between field stars and cluster stars. She should follow through on this point and talk to her Kitt Peak colleague Helmut Abt⁹ who has shown that NGC 7789 has an excess of 'blue straggler' stars which are supposed to be indicative of binary stars, since that is the only way to understand their colour and luminosity. The possibility that stars transfer mass before Li depletion occurs may be a straightforward explanation for the lack of Li depletion in the cluster.

It is clear that modern Li studies are very active and are not only telling us about cosmology and cosmic rays, but are also probing the details of internal stellar structure and evolution. $\square$

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Biominerals

Controlled crystallization

from J. C. Elliott

CRYSTALS that grow within or around an organism as a result of normal physiological processes are almost always of uniform size and shape, have a well-defined orientation and are deposited in a specific site. How this is achieved is for the most part a puzzle (see *Biological Mineralization and Demineralization*, ed. Nancollas, G. H.; Berlin, 1982). L. Addadi and S. Weiner (*Proc. natn. Acad. Sci. U.S.A.* **82**, 4110; 1985) have now provided insight into these processes by the discovery of an atomic-scale mechanism for the control of crystal shape and orientation by means of proteins that are rich in aspartic acid.

The atomic structures of most of the 30 or so mineral crystals that occur in the animal and vegetable kingdoms are known, often in considerable detail. Similarly, there is an enormous body of detailed information about the structure, synthesis and function of biological organic molecules and systems. Yet understanding the processes that take place at the interface between these

biominerals and organic molecules (and sometimes inorganic ions) that result in controlled mineralization has proved to be elusive. Often there is clear evidence of a close relationship between a biological molecule and its associated crystal — in bone many of the calcium phosphate crystals are located close to and even within collagen fibres, with their long directions (hexagonal axes) parallel to the length of the collagen fibres; and in some classes of mollusc shells the aragonite crystals (one form of calcium carbonate) are oriented with respect to the β -sheet of the protein molecules. But in other tissues, such as developing dental enamel, there is a clear orientation of calcium phosphate crystals but little evidence of an oriented protein matrix. Nevertheless, the concept that the orientation of mineral crystals can be controlled by an organic matrix is well established. If the orientation can be controlled by the matrix, it is a short step to presume that the matrix can sometimes initiate crystal

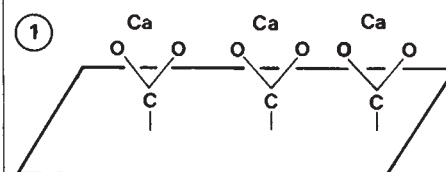
nucleation.

It is well known that some small molecules or ions can have a profound effect on the shape and rate of crystal precipitation at concentrations as low as 10^{-5} – 10^{-8} molar. The first indication that the same phenomenon might have biological relevance came from studies of naturally occurring inhibitors of stone formation in urine (for example, citrate, pyrophosphate and magnesium ions). The identification of pyrophosphate as a naturally occurring inhibitor led to the speculation that its local concentration could provide a mechanism for controlling crystal deposition in bone.

More recently, interest has centred on the role of acidic macromolecules, particularly proteins with carboxyl and/or phosphate groups in their side chains that can bind calcium ions and which are often found at sites where calcium phosphate or carbonates are being precipitated. In their elegant investigations, Addadi and Weiner study the effect of aspartic acid or serine-rich protein isolated from mollusc shells on the development of the crystal faces of calcium salts of various dicarboxylic acids and calcite.

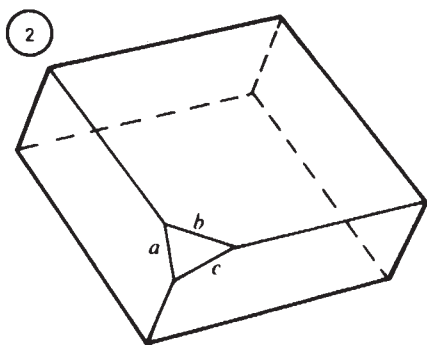
By not tackling the medically more relevant effect of inhibitors on the nucleation and growth of calcium phosphates, Addadi and Weiner have gained two advantages: first, they avoid complications that arise from the fact that there are several different calcium phosphates that are liable to precipitate and subsequently interconvert, unless conditions are very closely controlled; and second, calcium carbonate, as distinct from the calcium phosphate of bone, forms crystals large enough for the relative development of the faces to be determined without too much difficulty. Thus, they can identify the crystal faces that adsorb the inhibitor molecules because their growth is suppressed.

It was then possible, from a study of the structures of the compounds, to seek common structural features in the crystal faces that could be interacting with the adsorbed molecules. Addadi and Weiner find that the only faces of the calcium dicarboxylic acid salts affected are those that have carboxylate groups emerging perpendicular to the surface (Fig.1).



Furthermore, of the inhibitors studied, only those with a β -sheet conformation (aspartic acid rich matrix protein and polyaspartate) have a specific effect. It was deduced that there is a specific cooperative stereochemical interaction which involves the replacement of calcium ions bound to a rigid protein by those in

the surface of the crystal face which, ideally, would then occupy the same positions.



The planar carbonate ions in calcite are arranged so that their oxygen atoms lie in sets of parallel planes with calcium ions sandwiched between them. Thus, it would be expected that any specific stereochemical interaction between calcium-loaded aspartic acid rich proteins is on these planes. Indeed, this was found to be the case, but through a curious phenomenon: namely the development of a single face parallel to the planes of the carbonate ion (the plane *abc* in Fig. 2) when protein is present, even though the internal symmetry of the crystal requires that a diametrically opposite face should also develop.

Subsequent experiments showed that the crystals develop this single extra face because they are nucleated on acidic proteins that have been adsorbed on a flat surface of the container. The crystals then grow out of this face and develop normally with rhombohedral faces.

It is very satisfying that a detailed mechanism has been elucidated for the nucleation and control of the orientation of calcite crystals in molluscs, but how universal is this type of mechanism likely to be? Given the large variety of minerals and types of situations in which they can occur, it is probable that there are several ways in which deposition of minerals can be controlled. For example, crystal growth can be inhibited by adsorption of molecules onto screw-dislocations, and there is good evidence that in some forms of vertebrate hard tissues initial precipitation of calcium phosphates occurs inside cells, possibly in mitochondria. In addition it has been suggested that membrane-bound bodies derived from cells (the so-called matrix vesicles) are the sites of initial mineral precipitation. □

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Optimal foraging

When the going gets tough

from Paul H. Harvey

THERE are times of the day or, in seasonal environments, times of the year, when food is hard to obtain. During these difficult periods, animals may rest, sleep or hibernate. Alternatively, they may keep on feeding, though sometimes widening their dietary range. What interplay of selective forces determines the best response to food deprivation? A number of recent studies provide some of the answers.

The ways morphological structures, physiological processes and, ultimately, behavioural responses scale with body size provide a useful perspective to the problem. Across the animal kingdom, basal metabolic needs scale with the 0.75 power of body size, which means that larger animals need to use less energy per unit body weight to maintain themselves. Fat stores, however, increase with the 1.19 power of body size¹, so that larger animals can live longer on their stored reserves. For example, according to a recent paper by Stan Linstedt and Mark Boyce², the maximum time between fuelings for an elephant is about 200 times longer than for a mouse. The fasting-endurance difference becomes even greater at lower ambient temperatures, partly because larger animals are better insulated. Added to these advantages of large size gut volumes increase proportionately

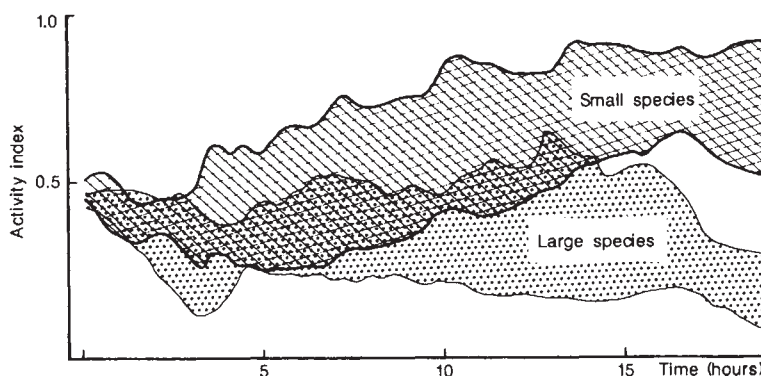
with body size and throughput times decrease¹. This means that larger animals are normally able to survive longer before they even start to call on stored reserves.

These differences in energy needs and available resources dictate different optimal responses to what Ilkka Hanski has termed an energy crisis. For example, if a shrew is deprived of food, should it rest until the crisis is over, or should it expend energy searching for scarce resources? The answer depends critically on both body size and the usual period that a shrew might be deprived of food in the wild:

Taking expected food deprivation times as constant across shrew species, Hanski reasons that a small shrew could survive only very short periods on a diet that contains a mere five per cent fewer calories than it needs to maintain itself. On the other hand a larger shrew, with sufficient energy reserves to weather the crisis, could rest until times are better.

Hanski's predictions were confirmed in an elegantly designed experiment comparing two large species (*Sorex araneus* and *S. isodon*) with two small species (*S. minutus* and *S. caucutiens*). The CO₂ output of a shrew in a metabolic chamber was continuously monitored by an infra-red gas analyser to measure rates of metabolism. The results were fed into a microcomputer that automatically released just enough food into the chamber to match energy expenditure, and the proportion of time the shrew was active was used as its 'activity index'. After 24 hours, the machine started feeding the shrew at a level such that, if it maintained its previous activity index, it would lose five per cent of its current body weight. After 15 hours of deprivation, individuals of the small species had appreciably increased their activity levels while the large species spent more time resting (see figure).

The importance of death from starvation among birds as well as small mammals has been known for some time although classical optimal foraging models have not considered the problem. The decision about whether to accept or reject a food item has been implicitly assumed not to depend on the forager's energy reserves. There is evidence from the pigeon *Columbia livia*³ and the great tit *Parus major* that birds become less selective in food-choice tests as energy reserves fall. Recent work makes a start towards incorporating food deprivation as a variable in optimal foraging models^{4,7}. The authors treat various cases in which animals encounter two types of prey with different energetic values and handling times. One model considers a continuous forager, like Hanski's small shrews; a second, more realistic, model adds short but unpredictable periods of food shortage to the first; and a third considers a species in which



Large shrews become less active whereas small shrews become more active when deprived of food. The results of this experiment by Hanski were predicted by the different ways metabolic needs and resource availability relate to body size (from ref. 3).

foraging stops predictably each evening, like a passerine bird feeding in winter. In accord with the available data, the models predict that animals should sometimes be less selective in their diet than the classical theory predicts. Furthermore, again agreeing with the data, the models show that optimal foraging strategies are expected to depend on current energy reserves, apart from the rather unrealistic case of a continuous forager encountering prey according to a Poisson process.

In essence, these new models add risk-sensitivity to foraging theory, and they draw attention to the need for a foraging animal to be prepared for periods of food shortage. This may sometimes be achieved by adopting a feeding strategy that gives a lower average food intake because it is associated with a lower variance in reward rate. A high variance in reward rate often should be avoided because available food reserves might not be sufficient for a period of food deprivation. One series of experiments provided common shrews with separate feeding stations that

had the same average reward rates, but with different variances. Just as predicted, the animals preferred to visit the less variable station⁹.

Once again a combination of theoretical, experimental and comparative approaches has provided useful and complementary insights into behavioural strategies. □

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Solid state physics

Magnetic interactions in quasi-two-dimensional systems

from J. J. Quinn

INTEREST in the problem of diamagnetic interactions among conduction electrons in solids has been revived by a recent observation of strong magnetic interactions in a stage-2 Br₂ graphite intercalation compound¹. In this material a layer of Br is sandwiched between every second pair of carbon layers. The magnitude of the interactions is sufficiently large, and the structure in the line-shapes sufficiently sharp, that the possibility of studying domain wall dynamics seems promising. The strength of the magnetic interactions in this material is undoubtedly a result of the almost two-dimensional nature of the constant energy surface in wave-vector space, which causes an entire Landau level to pass through the Fermi surface at the same value of the applied magnetic field. Although this leads to a very large number of electrons contributing to the oscillations, the result is still somewhat surprising because the highly oriented pyrolytic graphite sample is not a high quality single crystal.

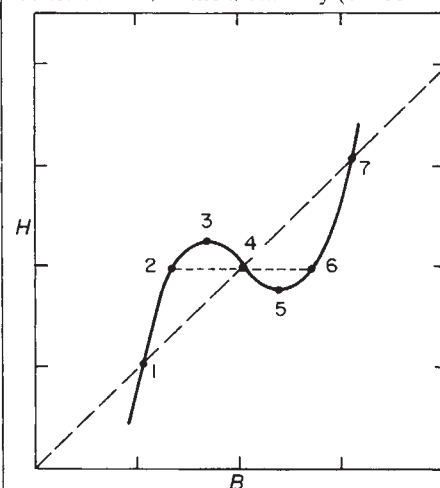
Magnetic interactions of this type were first recorded by Shoenberg² in studies of the de Haas-van Alphen effect in silver. He observed a surprisingly large harmonic content in the oscillations of the magnetization versus magnetic field in a region of temperature in which the simple equation $M = M_{ni}(H) = M_0 \sin(2\pi f/H)$ should have been valid. Here H is the magnetic field strength, M_0 is the maximum amplitude of an oscillation and f is the frequen-

cy. (For a two-dimensional electron gas $f = (hc/e)n/2$, the ratio of a quantum of flux (hc/e) to twice the area per electron n^{-1} .) Shoenberg conjectured that this simple equation, appropriate for a system of non-interacting electrons (hence the subscript NI), should be modified to account for the magnetization produced by the motion of the other electrons. The mean field expression for the magnetization of the interacting system (subscript I) is $M = M_I(H) = M_{ni}(B)$, where $B = H + 4\pi M$. Although H is typically ten thousand times larger than M , the frequency f can be sufficiently large (25,000 tesla in silver) that the small difference between B and H can completely change the phase of the sine function. However, beryllium and the noble metals were the only materials that displayed strong magnetic interaction effects at reasonable magnetic fields and temperatures, and the flurry of post-Shoenberg activity, both experimental and theoretical, slowed almost to a halt more than a decade ago. The recent observation of strong magnetic interaction effects in a quasi-two-dimensional material has caused renewed interest.

In the equation $M = M_{ni}(H + 4\pi M)$, M is a multiple-valued function of H when $8\pi^2 M_0 f/H^2 > 1$. This can be seen in the figure where $H = B - 4\pi M(B)$ is plotted as a function of B . For the part of the curve lying between points 3 and 5, $\partial B/\partial H$ is negative which is thermodynamically

unstable. If, however, we change M by varying the current in the coils of our magnet, the system never arrives at point 3. As the value of H is increased, the magnetization makes an abrupt change from the value at 2 to that at 6.

This behaviour can be understood by noting that the path of lowest free energy is 1-2-6-7. The behaviour described above is based on the implicit assumption that the sample is a long thin rod oriented parallel to the applied field (then, the magnetic field strength inside the solid is equal to H_0 , the extended magnetic field produced by the magnet). For the case of a flat disc, Condon³ suggested that when the system arrived at point 2 a slight increase in the magnetic induction would lead to the formation of a domain of magnetization corresponding to point 6. In other words, the region $B_0 < B < B_0$ would be a two-phase region in which H would be constant. One region would have magnetization M_0 , the other M_0 , the two stable values of the magnetization at that value of H . As B is increased, the amount of phase M_0 increases at the expense of phase M_0 . The argument is analogous to the solution, using Maxwell's construction, of the instability ($\partial V/\partial P > 0$)



A schematic of H , the magnetic field strength in the sample, as a function of $B = H + 4\pi M$, the magnetic induction, in the region where M is a multiple-valued function of H . The sequence 1-2-3-4-5-6-7 traces out one cycle of the de Haas-van Alphen oscillation. Between points 3 and 5, $\partial B/\partial H < 0$ indicating a thermodynamic instability.

in the Van der Waals equation for a gas-liquid transition. In both cases the instability is due to the implicit assumption of the existence of one homogeneous phase, and the solution is the recognition that several coexisting phases can have lower free energy. The region separating the two stable phases is a domain wall. The positive domain wall energy and domain wall thickness can be calculated⁴ in terms of the differential magnetic susceptibility and the cyclotron radius. The number and size of the domains at a given value of B results from a competition between the energy increase required to make an additional

domain wall and the decrease in emergence energy' resulting from the smaller magnetic energy density in a sample with a greater number of domains.

As the external magnetic field H is varied in the domain state, the average magnetization within the sample changes by the motion of domain walls. A small a.c. magnetic field should produce an oscillatory motion of the domain wall. The measurements¹, performed using a field modulation technique, show sharp structure. The possibility of probing the dynamics of the domain wall motion, through such measurements, is the most exciting aspect of the recent work.

A few additional points are worth mentioning. In stage-2 Br₂ graphite intercalation compounds, the frequency f is in a particularly favourable range¹ of about 200 tesla. Values of f in the range 20 to 200 tesla are readily achievable in some semiconducting superlattices. These artificially structured materials, like GaAs - GaAlAs or InAs - GaSb, are of great current interest. The fact that magnetic interactions should pin the Fermi level between Landau levels in a quasi-two-dimensional system has already been pointed out by Vagner *et al.*². This should lead to quantum Hall effect behaviour of

the magnetoresistance.

Once the mean field approximation $M(\mathbf{r}) = M_0(H + 4\pi M(\mathbf{r}))$ is used, it is not necessary for M to be spatially constant just because H is. In fact, the domain state is a simple manifestation of this. It corresponds to an instability in the wave-vector dependent³ differential susceptibility χ_q at $\mathbf{q} = 0$. In some systems the instability occurs for a finite value of $\mathbf{q}$, in which case a magnetization density wave results. Artificially structured semiconductor superlattices of many different types can be envisioned. As molecular beam epitaxy of these materials continues to develop, one can hope to fabricate other quasi-two-dimensional structures with large magnetic interaction effects and thus create a variety of new magnetic states. □

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Oncogenes

Growth factors short-circuited

from Kathleen Kelly

THE fundamental role of growth factors in controlling the normal proliferation of cells is well established. Some oncogenes may short-circuit the regulatory action of growth factors by encoding for products that resemble either growth factors or their receptors. Cell cultures that normally depend on external sources of growth factor to maintain proliferation may lose this requirement if they are infected by a retrovirus carrying an oncogene. The paper by Rapp *et al.*¹ on page 434 of this issue and two recent articles in *Cell*^{2,3} provide further evidence for the mechanisms that may be involved.

Rapp and colleagues show that two immortalized murine haematopoietic cell lines infected with retroviruses carrying the avian *v-myc* oncogene are transformed to grow continuously and independently of extrinsic growth factors. One is a T-killer cell line (CTB6) that, before infection, is dependent on interleukin 2 (IL-2) for growth, the other a line (FD-P1) that requires interleukin 3 (IL-3) or, alternatively, granulocyte-macrophage colony-stimulating factor (GM-CSF). Similarly, the other two studies^{2,3} show that both FD cells and IL-3-dependent mast cells derived from bone marrow lose their stringent requirement for growth factor following infection by Abelson murine leukaemia virus. The *v-abl* oncogene of

that virus encodes a membrane-associated molecule and belongs to a family of oncogenes encoding tyrosine kinases.

What is the mechanism by which these transforming retroviruses relieve the requirement for exogenous growth factors? Previous examples demonstrating the induction of secreted growth factors by transforming-retrovirus infection might suggest an autocrine stimulatory mechanism^{4,5}, in which the cells are enabled to produce growth factor that stimulates their own receptors. Interestingly, these *v-abl* transformed haematopoietic cells did not appear to be synthesizing or secreting the relevant growth factors, and changes in their receptors for IL-3 and GM-CSF were not detected by radioligand binding, indicating there is neither an obvious blockade of receptors nor unusual increase in receptor numbers. CTB6 and FD-P1 cells expressing *v-myc* secrete growth factors that can be detected by bioassay of conditioned media.

How then do *v-abl* and *v-myc* act in these haematopoietic cells to overcome the normal interaction between growth factor and receptor required for growth? The answer is unknown, but the following points deserve consideration. Because a number of growth factors stimulate a tyrosine kinase activity in their receptors, one might predict that the chain of events

following IL-3 or GM-CSF receptor binding also includes the activation of a membrane-associated tyrosine kinase. This activation might either be direct, should the tyrosine kinase form part of the receptor molecule or a receptor complex, or indirect through intervening steps. The role of such a kinase may then be superceded in a deregulated manner by the *v-abl* product. In fact, *v-abl* may itself be a homologue of a growth-factor receptor gene, analogous to *v-erb B* and *v-fms*. Structure-function information about IL-3 and GM-CSF receptors is now needed to assess this possibility.

The action of *v-myc* in abrogating the dependence on growth factor presumably occurs in the nucleus at a site separated from the growth-factor receptors. Because the cellular *myc* gene, *c-myc*, is induced by growth agents, it has been suggested that it is an intracellular mediator of mitogenesis. Immortalized murine or rat fibroblasts transfected with *c-myc* or *v-myc* are not, however, made completely independent of growth factor^{6,7}. Consequently, it seems that *myc* sequences, at least in fibroblasts, must work in concert with other intracellular mediators to stimulate continuous proliferation in the absence of exogenous growth factors. Possibly CTB6 and FD-P1 cells have quite different growth regulation requirements from fibroblasts. But *v-myc* expression in FD-P1 and CTB6 cells is very high, over 100-fold greater than normally expressed *c-myc*, whereas the transfected fibroblasts have considerably lower *myc* RNA levels; this may account for the difference. It is interesting that the cells infected with *myc*-carrying viruses do not express *c-myc*, whether growth factors are present or not, suggesting that *c-myc* is negatively regulated when *v-myc* expression is excessively high.

By using defined cell types whose growth requirements are known, the three articles discussed here have begun to address elegantly the mechanisms by which *abl* and *myc*-carrying viruses transform cells. The entire process of *in vivo* tumour induction cannot, however, be extrapolated from *in vitro* systems, because the adaption of cells to culture probably involves genetic changes. In fact, due to the low frequency with which retroviruses infect these haematopoietic cells, it is not clear what percentage of virally infected cells in the studies have become growth factor independent. Even so, understanding the complex issue of retroviral transformation has come one step closer. □

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The seeds of agriculture

Robert Chapman

The Neolithic Transition and the Genetics of Populations in Europe.

By Albert J. Ammerman and L.L. Cavalli-Sforza.

Princeton University Press: 1985. Pp.176. \$25, £18.

Neolithic Europe: A Survey. By Alasdair Whittle.

Cambridge University Press: 1985. Pp.363. Hbk £27.50, \$44.50; pbk £9.95, \$17.95.

Prehistoric Farming in Europe. By Graeme Barker.

Cambridge University Press: 1985. Pp.327. Hbk £27.50, \$44.50; pbk £9.95, \$14.95.

THE advent and adoption of agriculture have been viewed consistently as marking a threshold in human evolution. Since the middle of the nineteenth century, archaeologists and anthropologists have considered agriculture as providing the essential foundation for the emergence of social differentiation and specialization, cultural complexity and urbanism. After the adoption of agriculture, hunters and gatherers rapidly gave way to more complex, sedentary societies, which in areas such as the Near East and Mesoamerica developed into the world's first states. Attempts to explain agricultural origins have been environmental and cultural, monocausal and multicausal, testable and untestable. Faunal, floral and cultural evidence has been collected at increasing rates, either to support or refute these explanations.

In Europe the study of the origins of agricultural societies, and their subsequent development, has benefited from over a century of data collection and analysis. According to the diffusionist model, the spread of plant and animal domestication, as well as technological and social changes, could be traced through a process of diffusion from the Near East. Radiocarbon dating has led archaeologists to reject diffusion for the later technological and social changes of the Neolithic and Bronze Ages, but a Near Eastern origin for agriculture has remained a preferred interpretation. Is this justified? What processes of innovation and adoption took place in Europe? How can we explain the cultural evolution witnessed in the European Neolithic? These are some of the important questions addressed in recent archaeological research, as seen for example in publications by Colin Renfrew (among them *Problems in European Prehistory*; Edinburgh University Press, 1979) and Robin Dennell (*European Economic Prehistory*; Academic Press, 1983).

The diffusion model for European agriculture, from c.6000 to c.3500 BC, is adopted by Ammerman and Cavalli-Sforza in their highly original and thought-provoking book. Analysis of the earliest dates for cereals from archaeological sites across Europe suggested to them that there has been a constant rate of spread, from south-east to north-west, averaging

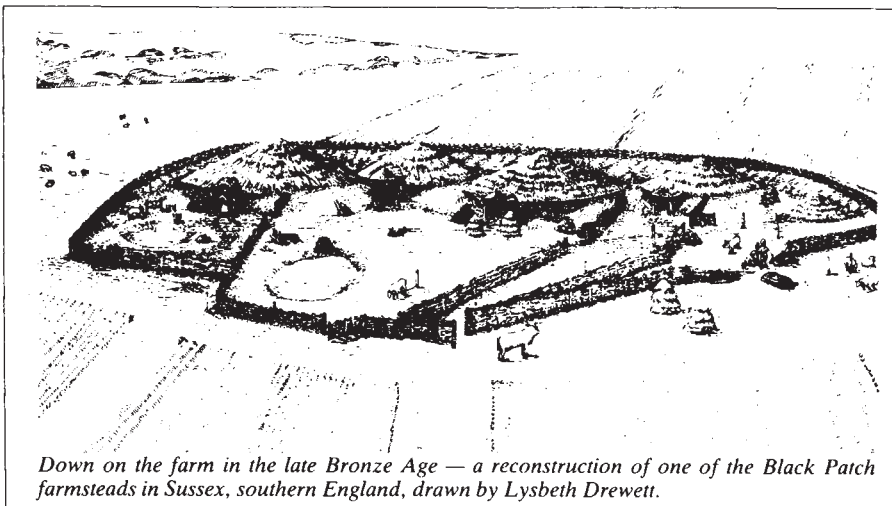
out at 1km per year. In addition there was regional variation, with areas such as central Europe and the west Mediterranean showing faster than average rates. The model used to account for these observations is one of demic diffusion (that is a physical expansion of population, rather than a diffusion of culture and economy through essentially stable, immobile populations), and the specific form taken by this process is referred to as a "wave of advance" model. This is derived from genetics and is delightfully simple: given the assumptions of logistic population growth and small-scale local population movement (for example through marriage), the model predicts a wave of population expansion from initial centres at a constant rate in all directions. As the authors claim, the model suggests "how local processes such as population growth can produce what in some respects is a form of colonization without colonists". The initial test of the model, with a number of specified growth and migration rates, produces a wave of advance closely comparable to that observed from the archaeological data.

In addition the authors argue that demic diffusion would have had implications for the distribution of gene types within Europe, the movement of population from south-east to north-west (and interbreeding with local hunter-gatherer populations) producing a cline of genetic similarity. In the absence of information on prehistoric gene frequencies, the au-

thors analyse modern gene systems and, surprisingly, reproduce a pattern comparable to that predicted. The implication that "major patterns detected in synthetic gene maps probably have meaningful histories behind them" is of great importance and suggests a fruitful area for future cooperative research by archaeologists and geneticists.

Overall Ammerman and Cavalli-Sforza have presented one of the strongest and clearest arguments for the demic diffusion model for European agricultural origins. But can we accept it? The evidence collected in recent years for intensification towards agriculture in the later Mesolithic is little discussed, nor indeed is the context of adoption as opposed to innovation in subsistence economies. Communities did not accept agriculture just because it was there. The swidden model (long fallow cultivation combined with frequent settlement relocation) for agricultural dispersal has been severely criticized in recent years and the implications of the alternative model of fixed plot cultivation are not considered. Further the cultural evidence for continuity in the west Mediterranean is directly contrary to a demic diffusion model.

All of these objections are contained in Alasdair Whittle's "wide-ranging interpretative introduction" to *Neolithic Europe*, though he does not cite any papers by Ammerman and Cavalli-Sforza. Whereas the latter concentrate on the detailed application of one model to agricultural innovation and colonization, Whittle has written a detailed and commendable synthesis of colonization and cultural change from c.8000 to c.2000 BC, from late hunters and gatherers to metal-using, hierarchical societies. There is much useful detail on the basic archaeological record of different areas and periods, and this will be plumbed with profit by students. In contrast to Ammerman and Cavalli-Sforza, Whittle believes that, in order to explain cultural change in the past, we must focus on the determining role of the society in accepting or rejecting



Down on the farm in the late Bronze Age — a reconstruction of one of the Black Patch farmsteads in Sussex, southern England, drawn by Lysbeth Drewett.

innovations, as well as in provoking organizational changes. There is much reference to "social tensions" and "contradictions", to societies as composed of conscious actors rather than "passive recipients". What is seen as demographic or environmental determinism is rejected. The roots of this approach lie in structural-Marxism.

Although I find much to admire in Whittle's book, and recommend it to be read and consulted, I have three main objections to his approach. First there is no allowance for the unintentional consequences of human actions, surely a necessary component of any theory of human evolution. Secondly there is no recognition of the different time-scales over which cultural changes take place: changes which are apparent over several hundred years of the archaeological record may have been imperceptible to individuals. This gives rise to questions of proximate as opposed to ultimate causality. Lastly there is the archaeological record itself, and what we make of it. There are continual references to the difficulties posed by drawing social inferences from settlement or burial data, to the possibilities of social differences being "masked" by communal ideologies. But unless we have the methods to identify social variables, surely the theoretical approach exemplified in this book will struggle to get off the ground?

A rather different approach, and one with which Whittle would presumably disagree, is presented in Graeme Barker's book, the first synthesis of information on prehistoric farming in Europe since Grahame Clark's *Prehistoric Europe — The Economic Basis* (Methuen, 1952). The amount of new data (on settlements, fauna and flora, and on prehistoric environments) collected since that date is truly astonishing. This alone marks out *Prehistoric Farming in Europe* as a book which will join Whittle's on all students' reading lists.

Barker's approach to this evidence is to try and isolate locally varying economies, even within single regions. No longer is prehistoric Europe peopled by a series of cultures, each with their own distinctive economies. Subsistence economies are viewed as local adaptations, with the model of agricultural colonization (as supported by Ammerman and Cavalli-Sforza) being rejected firmly for every area of Europe in turn. But why do subsistence economies change? Barker follows D.B. Grigg (*The Dynamics of Agricultural Change*; Hutchinson, 1982) in proposing four major stimuli: climatic and environmental change, technological change, population increase and social organization. While Whittle would champion the last of these, Barker argues for a combination of environmental and demographic stimuli. It would be unfair to stigmatize these different views as social versus environmental determinism. The

fact that radically opposed interpretations are proposed for the same data is very revealing of the current state of archaeology.

There are important lessons to be learnt from all three of these books. Even in an area as long researched as Europe, there is still a need for rigorous model-building (as in Ammerman and Cavalli-Sforza's work) and scholarly, provocative synthesis (as provided by Whittle and Barker). There is still much to be learnt about agricultural origins and the evolution of agricultural societies in Europe. Opposing theories are welcome, as long as they are relevant to the scale and character of the archaeological record. Ultimately it is our ability to interpret archaeological data in terms of variables relevant to our theories that will ensure progress in our knowledge of the past. □

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Bird organization

John Andrews

A Dictionary of Birds. Edited by Bruce Campbell and Elizabeth Lack. *Poyser/Buteo*: 1985. Pp.670. £39, \$75.

The Encyclopaedia of Birds. Edited by Christopher M. Perrins and Alex L.A. Middleton. *George Allen & Unwin/Facts on File*: 1985. Pp.445. £25, \$35.

ELEVEN years ago I obtained, by slightly dishonourable means, a copy of Landsborough Thomson's *A New Dictionary of Birds*, then a decade old and out of print. Soon, my conscience stopped pricking me: the book was too useful to sustain a sense of guilt at its mode of acquisition. Time passed. The text became dated and I cannot recall when I last referred to it.

The publication of *A Dictionary of Birds* is thus doubly welcome, being a legitimate acquisition which updates and expands the earlier work. Running to over a million words, contributed by some 280 ornithologists and other specialists, it incorporates a wealth of new fact and interpretation. Like its predecessor, it comprises articles on general subjects relating to birds, and on different kinds of birds treated by families, all of which are covered. There are also short entries defining special terms. The whole work is arranged alphabetically.

The English names of birds chosen as entry headings are those used in Britain but North American names and others used in the English-speaking world have been included as far as possible. Scientific names of groups above generic level are also listed, cross-referring to the vernacular.

Overall, the text is splendidly comprehensive and comprehensible. Though cer-

tainly a serious reference work it does not omit offbeat aspects — for instance birds in art, birds in music and heraldic birds all get extensive consideration along the way from abdomen to zygomatic arch. My only serious criticism concerns the illustrations. The black-and-white photographs are helpful and informative, but the line drawings, many of which are "bird-on-a-stick" pictures, are disappointing and fail to complement the text.

By contrast, the illustrations in *The Encyclopaedia of Birds* are one of its major strengths. Most of them are colour photographs, all of good quality and often of real beauty: there is a pleasing dearth of nest shots and instead many depict infrequently seen aspects of behaviour or physical skills. Supplementing these are colour paintings and line drawings by several acknowledged bird illustrators, extending the range of species covered and the activities portrayed.

The aim of this book is to provide a succinct, up-to-date account of the world's birds. The text, like that of the *Dictionary*, consists of a series of articles by specialist contributors — 90 in this case — dealing with a single family or group of closely related families, the whole arranged in systematic order, and each article covering special physical adaptations, distribution, evolutionary history, classification, breeding, diet and feeding behaviour, social dynamics and spatial organization, conservation and relations with man. An "information panel" — not as gimmicky as it sounds — precedes the text on each family, giving a map of its world distribution and other basic information, and listing all its members. A general introduction discusses classification and the structure and adaptations of birds, and there is a well chosen bibliography and an index to species.

Much of the material is here receiving its first airing in the popular literature: it will be interesting to watch its progression into other, less original works, in the process gradually losing precision or, to judge by some past examples, even comprehensibility. Perhaps surprisingly, most of the expert contributors write well, or were very ably edited, for which Drs Perrins and Middleton should take much credit. Likewise the publisher's production team should share in the congratulations: it is easy to dismiss glossy and abundantly illustrated books as lightweight, but this volume represents a successful union of science and art and has plenty to offer both the armchair birdlover and the serious ornithologist.

Comparisons are odious and both books have a great deal to offer. But, if forced to choose, I would regard the *Encyclopaedia* as desirable and the *Dictionary* as necessary. □

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Cases of pollution through time

Peter D. Moore

Historical Monitoring. MARC Report No. 31. Monitoring and Assessment Research Centre, The Octagon Building, 459A Fulham Road, London SW10 0QX, UK: 1985. Pp.323. Pbk £20, \$30.

SINCE the development of the concept of Uniformitarianism in the eighteenth century it has been the common practice of stratigraphic geologists and palaeontologists to use the present as a key to the past. Only in relatively recent times has it been recognized that many modern environmental problems can better be understood if their historical perspective is adequately appreciated. So it is that the techniques of biology, physics and chemistry are now being applied extensively to the study of the temporal development of many of our current ecological crises, from heavy metal pollution to acid rain and the greenhouse effect.

This report from the Monitoring and Assessment Research Centre of Chelsea College, London, is a review of the many areas in which such studies have been made and an assessment of the relative values of the various approaches. It is not a practical manual with detailed recipes of methods, but it provides a thorough bibliography through which the full information on such methods can easily be located.

The introduction of a time dimension into pollution studies demands that materials are available for analysis which have developed or accumulated in chronological sequence. Five main forms of evidence are described here — marine and lake sediments, ice masses, peats, tree rings, and animal and plant remains — mainly as found in museums, but also including archaeological remains, particularly human tissues. Each is discussed and case studies are described to illustrate their respective potential as sources of historical information.

The text is assembled in an orderly, yet lively and critical manner so that the reader is constantly reminded of the complications inherent in the interpretation of data from any of these materials. Does the concentration of a particular pollutant, for example, reflect the ambient level at the time of its formation? Can one separate the natural background noise of an element from long-term trends produced by human influence on global cycles? Often the answer is negative. Take sulphur in the Antarctic ice cores: here there is a considerable input from marine sulphur sources, such as vents, and high variability is also introduced by terrestrial volcanic eruption. In combination, these problems make it impossible to detect long-term

trends in anthropogenic atmospheric sulphur in ice cores.

The use of peat cores for documenting fallout of heavy metals, such as lead, has been dogged by problems of metal movement within the profile. Living plants may transport the element down through their roots, and so upset its stratification, or the metal may be washed through loosely compacted surface layers and then accumulate at the water table. Nevertheless, lead profiles in peats do seem to correlate well with the evidence from historical records.

The bones of our ancestors may also indicate former pollution levels, but are some elements preferentially absorbed from or lost to the soil after burial? Could

the fact that Peruvian bones of 1300 AD have only one-tenth of the lead levels of modern human bones be due to such post-interment processes? Because most other metal contents have remained the same, this seems unlikely.

This report is a concentrated collation of material which both conveys the results of current research and provides the reader with sufficiently critical opinions for the appraisal of its reliability. It is an ideal source to which the advanced undergraduate and postgraduate student can refer as well as the research worker. □

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Modified responses

Ruth Arnon

Immune Modulation Agents and Their Mechanisms. Edited by Richard L. Fenichel and Michael A. Chirigos. Dekker:1985. Pp.678. \$99.50 (North America), \$119.25, SwFr.275 (elsewhere).

IMMUNOMODULATION is a relatively new term which is used to describe two different phenomena — the potentiation and the suppression of the immune response. The past decade has witnessed the discovery of an ever-increasing number of natural and synthetic compounds which have interesting immunostimulant properties, while, at the same time, progress in basic cellular immunology is revealing numerous and diverse regulatory materials produced by the immune system itself. None of these latter products are antigen-specific, but they do have the advantage of acting physiologically and are usually of relatively low toxicity. Several immunomodulation agents, such as prostaglandin, cyclosporin A and thymic factors, have already found clinical application, but we still have only a limited understanding of their mode of action.

Immune Modulation Agents and Their Mechanisms, a recent addition to Dekker's *Immunology* series, attempts to fill in the gap in the literature on this important group of products. The book contains an impressive list of biological response modifiers, description of their properties, a review of the relevant literature and a summary of the clinical trials performed with them. Its advantage, mainly for clinicians and immunopharmacologists, is the gathering of all this information under one roof, thus supplying the reader with an overview of the various immunomodulators and a basis for comparison between them. On the other hand, the parts dealing with mechanisms of action are somewhat scanty. True, this may be the only mechanistic evidence that is available, but the title of the book promises more than is delivered.

Several of the contributions are excellent — Mathé's account of immunomodulating agents is concise but informative; the two chapters on thymosin and that on cyclosporin A are comprehensive, and try to explain mode of action as well as to supply the reader with all the available information; and the chapters on autoimmune diseases and on tumour and metastatic diseases are also very good. Regrettably, however, this high level is not uniformly maintained throughout the book.

A few other shortcomings are also evident. Each chapter deals separately with a different agent, hence there is a degree of repetition of general material. Many of the agents are discussed in two separate contributions in Parts I and II of the book; this division is not clear cut and the result is considerable redundancy in some pairs of chapters such as 1 and 29, 3 and 23, 4 and 19, 5 and 22, 8 and 26, and 10 and 23. Also, the introduction to the book as a whole and those to its two constituent parts are too short and not very useful. A single, all-embracing summary of the field would have been preferable.

On the whole, however, this is a helpful book which provides a thorough survey of the literature in this burgeoning area of research. Clinicians, pharmacologists and immunologists will benefit greatly from reading it. □

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New in paperback

- *Realism and the Aim of Science*, Vol. 1 of the *Postscript to the Logic of Scientific Discovery*, by Karl R. Popper, edited by W.W. Bartley III. Publisher is Hutchinson, price £11.95. For review see *Nature* 302, 357 (1983).
- *Superforce: The Search for a Grand Unified Theory of Nature*, by Paul Davies. Publishers are Touchstone (an imprint of Simon & Schuster) and Counterpoint (Unwin Paperbacks), prices \$8.95, £3.95. For review see *Nature* 312, 787 (1984).
- *Betrayers of the Truth: Fraud and Deceit in Science*, by William Broad and Nicholas Wade. Publisher is Oxford University Press, price £3.95. For review see *Nature* 302, 774 (1983).

Buzzing around the intellect

R. S. Woolhouse

Leibniz: A Biography. By E. J. Aiton. Adam Hilger: 1985. Pp. 370. £29.50, \$55.

To Ramazzini, the father of industrial medicine, Gottfried Leibniz (1646–1716) was unsurpassed in the range of his intellectual accomplishments; to another contemporary, the philosopher Christian Thomasius, he was a living library. But it was his close friend, Sophie, Electress of Hanover, who best caught his style in comparing him to a bee, sucking honey everywhere he found it. Besides suggesting personal charm, the remark indicates the unremitting industry and persistence, the openness to stimulation from any quarter, and the imaginative eye for the constructive development of a chance idea, which characterized the life of this *universalgenie*. Like one of his metaphysical monads, he was a living mirror of the whole world. Active in diplomacy and politics, original in philosophy and mathematics, technologically inventive, he took a constructive interest in whatever came his way — be it law, geology, chemistry, Chinese writing, economics, industrial management, genealogy, public health or theology. Quite apart from his

purely intellectual and diplomatic activities, at one moment he might be at work on the construction of a calculating machine, chronometer or drainage pump, at another composing a poem to commemorate a birth or death, or designing a medal as a New Year present.

According to Bertrand Russell, Leibniz deliberately preferred a courtly life and the company of the socially great to an academic career. He has encouraged us to think of a man flawed, even held back, by somewhat worldly interests. It is true that Leibniz, having refused a professorship at the age of 21, was not long in acquiring the first of his distinguished noble patrons, Baron Johann von Boineburg. But it was his intellectual plans he thought could not come to fruition in a university, not his social aspirations. He made plain to his future employer, Duke Johann Friedrich of Hanover, that what he sought was time and financial support for his scientific and cultural projects. It is absolutely clear that he would have preferred to remain in the intellectual hot-house of Paris, with a paid appointment from the Academy of Sciences, than go to Hanover.

Russell has also encouraged the picture of Leibniz as a man whose talents were wasted and dissipated by being spread too thinly. But what emerges from E. J. Aiton's absorbingly extensive and detailed biography is not only the scale of some of Leibniz's ideas but also the far-reaching practical imagination and persistence with which he sought to carry them out. From early on in his schooldays, and throughout much of his life, he worked on the idea of a universal characteristic. The initial conception was of an alphabet of human thought, a universal language that would speak to the understanding rather than to the eye. By its means even such subjects such as metaphysics and ethics would be amenable to the same sort of proof as algebra and geometry. As a basis for demonstration and discovery of all truth the calculus would presuppose an encyclopaedia of all existing knowledge. As Leibniz began to see, the compilation of such an encyclopaedia, not to say the development of the calculus itself, required scholarly collaboration on a large scale. With this in mind he envisaged an Academy to carry it out and sought the help of Duke Friedrich, suggesting the use of the increased profits from the Harz mines drainage scheme he worked on. Nor was this the only scheme with which Leibniz persisted. Another was a plan for the reunification of the Churches, on which he worked both by way of political and diplomatic lobbying and by way of establishing its theoretical theological foundations. A third was the vast undertaking of the history of the House of Brunswick which was commissioned by Duke Ernst August of Hanover.

Even given that he seems to have lived only for work, it is astonishing that despite all of these endeavours, and despite the

diplomatic and administrative duties that came his way from his employers, Leibniz could have had time for the development of the ideas for which he is more famous. He seems to have had the happy knack of making the most of an occasion or of putting to good use any spare moment. Whether on board a boat waiting for the wind to change, or spending the night in an inn on some business journey, he would compose a mathematical paper or write one of his philosophical gems.

Aiton's biography, the first major one in English, tells of all Leibniz's multi-

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Leibniz — "Active in diplomacy and politics, original in philosophy and mathematics, technologically inventive ..."

farious activities. Also it very usefully explains and summarizes his ideas as expressed in his published and unpublished papers and correspondence. Those wanting a skilfully clear and concise account of anything from Leibniz's development of the differential calculus, through his attacks on Cartesian physics, to the metaphysics of substance and the pre-established harmony will find it here. They will also find much of interest from the point of view of intellectual history in general: the importance of such as Henry Oldenburg, Secretary of the Royal Society, as a clearing-house for ideas; the publication of mathematical results by formal registration and informal circulation; the foundation of scientific societies in the seventeenth century; and the increasing institutionalization and internationalization of science.

There is, however, not much of an explicit account of Leibniz the person. Aiton gives the facts and describes the ideas pertaining to the life of this great man. But he does not attempt any comment on or reconstruction of the personality whose life it was. We learn as a fact that Leibniz considered marriage on two occasions. We learn as facts of the evidently close friendships he had with the Electress Sophie and Queen Sophie Charlotte, and of his illness and depression on the death of the latter. But as to what should be made of them Aiton does not attempt to say. □

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The first human retroviruses have been discovered during the past six years. They cause two diseases which involve disturbances of the growth of the T4 lymphocyte, a remarkably specific target cell type. This cell, which is central to the regulation of the immune system, is induced by human T-lymphotropic virus type I (HTLV-I) to excessive proliferation (leukaemia) and by HTLV-III to premature death (acquired immune deficiency syndrome, AIDS). Both also seem to be indirectly involved in several other disorders. The genetic structures of these retroviruses and the mechanisms by which they usurp host-cell functions are novel among retroviruses.

HUMAN retrovirology is a relatively young field, even though the search for retroviruses as human pathogens dates back to the first association of this class of viruses with animal cancers at the beginning of this century (see ref. 1). So far, the only well-characterized human retroviruses belong to a single virus group possessing tropism for a subset of helper T lymphocytes. Because of this unique target cell specificity, these viruses, termed human T-lymphotropic viruses (HTLVs), destroy the immune system of the infected individual. Depending on the particular virus subgroup, this may lead to abnormal proliferation of functionally impaired helper T lymphocytes (leukaemia) or to depletion of the same target cell population (immunosuppression). This distinction is not entirely clear cut, however, as the leukaemia virus (HTLV-I) can also cause immunosuppression and opportunistic infections, and it is possible that the immunosuppressive virus (HTLV-III or lymphadenopathy-associated virus, LAV) can cause leukaemia once its cytopathic property is blocked. Nevertheless, it is now clear that HTLV-I and HTLV-III are the respective aetiological agents of an aggressive form of leukaemia, adult T-cell leukaemia (ATL), and AIDS. A third member of this family, HTLV-II, has been detected only rarely. Although it was first isolated from a patient with leukaemia, it has not yet been linked to any human disease. HTLV-I and HTLV-II seem to have recently stemmed from a common progenitor as there is extensive homology over their entire genomes and both have the capacity to transform T lymphocytes *in vitro*. HTLV-III has a genetic structure distinct from HTLV-I and HTLV-II and exhibits a profound cytopathic effect on cells infected *in vitro*. However, some common biological and biochemical properties characterize all three viruses. In addition to being exogenous human retroviruses, these include a remarkable tropism for the helper T lymphocytes possessing the cell-surface antigen OKT4; impairment of T-cell function; induction of syncytia (T cells) and formation of multinucleated giant cells in culture; a high relative molecular mass reverse transcriptase that prefers Mg^{2+} ; a small (p24/p25) major capsid protein; p24 cross-reactive antigenic determinants detected with either heterologous (rabbit) antisera or human monoclonal antibodies; juxtaposition of the p24 to the NH_2 -terminal gag protein (that is, absence of the proline-rich phosphoprotein in its usual position); similar modes of transmission (sexual, congenital and blood); a probable African origin; and infection of both man and some Old World monkeys. In addition, there are other structural and functional similarities of their genomes, some of which are unique among known retroviruses, which we describe here.

HTLV-I and ATL

Before the isolation of the first HTLV, we had developed sensitive assays for virus detection and *in vitro* culture systems for different types of haematopoietic cells. Following the discovery of reverse transcriptase by Temin² and Baltimore³, we could

optimize detection of low-level activity of this enzyme and distinguish it from cellular polymerases^{4,5}. In 1976, a growth factor specific for mature T cells (T-cell growth factor, TCGF, or interleukin-2, IL-2) was discovered in our laboratory⁶. This allowed the selective growth of different T-lymphocyte populations *in vitro* and has substantially changed many areas of T-cell research⁷. However, it was not until 1978 that the first human retrovirus was isolated from cultured T-lymphocytes of a patient in the United States with a mature T-cell malignancy; the first detailed characterization⁸⁻¹⁵ included an isolate from a second patient⁹. This virus, now designated HTLV-I, is the prototype for over 100 isolates of the same virus obtained from many parts of the world. Next to the discovery of the virus, the most important advance was the aetiological link of the virus to a disease. This link was not apparent from the early epidemiological studies of the sera from the US patients, as only a few samples were positive^{13,14}. However, advances in purification of viral proteins¹¹, preparation of hyperimmune¹¹ and monoclonal¹⁵ antibodies, as well as nucleic acid probes¹², were soon forthcoming. In 1980, we became aware of a syndrome called adult T-cell leukaemia (ATL), first described in Japan by Takatsuki and colleagues¹⁶, which clusters in certain parts of south-western Japan. As the leukaemic cells, like the tumour cells from the sporadic HTLV-I-positive cases in the United States, are OKT4⁺ lymphocytes, often with distinct morphology (for example, abnormally lobulated nuclei), we suspected that HTLV-I might be the cause of this disease cluster. We therefore examined coded sera provided by Y. Ito, N. Nakao, T. Aoki and their colleagues and found almost 100% of the sera samples from ATL patients to be positive^{17,18}. These data were presented at a workshop in 1981 where Y. Hinuma also presented immunofluorescence data on reactivity of sera of ATL patients with cell-surface antigens of a cell line established by Miyoshi and electron micrographs of this cell line (ref. 19). At the time, the 'Japanese' virus (then called ATL) was not yet characterized in detail, but subsequent studies soon proved that ATL and HTLV-I were identical^{20,21} and associated predominantly with a clinical entity typified by ATL. The first independent isolate of HTLV-I was obtained by Yoshida and colleagues²². Other endemic areas were soon uncovered (Fig. 1), including the Caribbean islands^{23,24}, the southeastern United States²⁵, parts of South America^{24,26}, parts of southern Italy²⁷ and many parts of Africa^{28,29}. The virus is also endemic in European emigrant populations from these areas, such as the Surinam population in Holland³⁰, the West Indian population in England²³ and the Ethiopian Jews in Israel¹⁴. A virus designated simian T-lymphotropic virus type I (STLV-I) has been identified in some Old World monkeys and it is particularly prevalent in African green monkeys^{29,31}. STLV-I is highly related (>95%) to HTLV-I³². Infection of animals with STLV-I is also associated with malignant lymphomas³³. Based on these and other observations, we postulated that HTLV-I originated in Africa and was brought

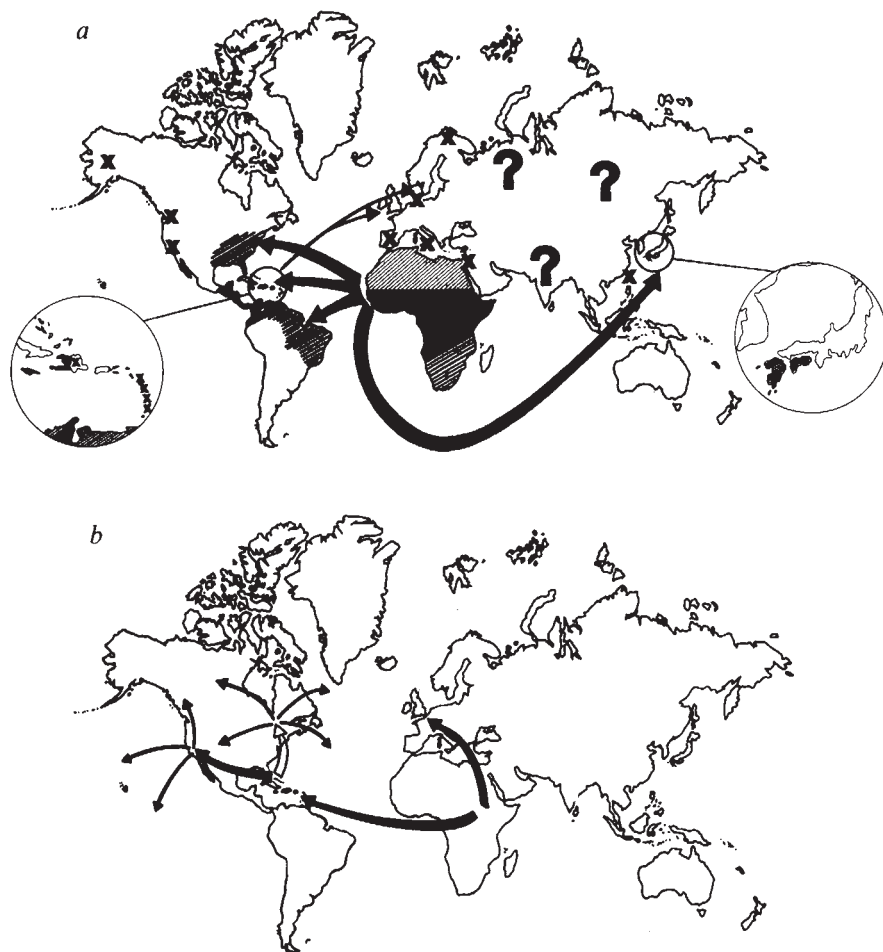


Fig. 1 Geographical distribution of HTLV-I and HTLV-III. *a*, Possible origin and prevalence of HTLV-I; *b*, Possible origin and spread of HTLV-III. *a*, Regions where the incidence of infection (seropositivity) are highly prevalent (>5%); □, moderate (1-5%). X, places where HTLV-I infection has been detected but extensive seroepidemiology has not been reported. ?, areas not studied. Insets are enlargements of two endemic areas, the Caribbean islands and Japan. *b*, Thick arrows indicate primary spread; thin arrows, secondary spread.

to the other endemic areas by commercial and slave trades dating back to the sixteenth century³⁴. Remarkably, Kanki and co-workers recently discovered a retrovirus (designated STLV-III) highly related to HTLV-III in African green monkeys³⁵ and macaques, and in the latter species, the virus is also associated with an AIDS-like disease. Natural infection of more than one species by the same or by a highly related retrovirus had not been demonstrated with any other retroviruses, yet it has now been found with both HTLV-I and HTLV-III. Thus, these findings represent yet another unique feature of the HTLVs and another common property of HTLV-I and HTLV-III.

The epidemiological results, the findings in monkeys, the *in vitro* transformation of primary human T cells and, most importantly, their molecular properties, led to the conclusion that HTLV-I is the primary cause of a human cancer. The major conclusions relating to the molecular biology studies from our laboratory and by M. Yoshida and co-workers, reviewed recently³⁶, are that: (1) all ATL cells contain an HTLV-I provirus (usually one copy), whereas normal cells generally do not; (2) the provirus is clonal, indicating that infection was before the time of the first transformation and not later as a passenger virus; (3) the integration sites, although the same in each cell of a tumour, vary from one patient to another. Therefore, transformation by HTLV-I (and bovine leukaemia virus, BLV) probably operates through a *trans* mechanism and not through long terminal repeat (LTR) activation of a nearby cellular gene.

HTLV-III and AIDS

The idea and the approach to the identification of a unique human T-lymphotropic retrovirus in AIDS is largely derived from epidemiological data and from studies on HTLV-I. The idea was first proposed by R.C.G., based on several considerations derived in part from discussions with M. Essex. First, epidemiological studies carried out chiefly by the Centers for

Disease Control in Atlanta, Georgia, particularly those pertaining to transmission of the disease by filtered factor VIII in blood transfusion cases strongly implicated a viral agent (see ref. 37 for review). Second, animal retroviruses such as feline leukaemia virus, were known to cause an AIDS-like disease as well as leukaemia³⁸. Third, the apparent defect in the helper T-lymphocyte population suggested a restricted tropism for the virus that was highly reminiscent of HTLV-I and HTLV-II. Fourth, the manner of transmission of HTLV-I was believed to be sexual, congenital and by blood contamination, routes expected from an AIDS agent. Fifth, the fact that HTLV-I and HTLV-II also showed immune suppressive activity *in vitro*³⁹⁻⁴¹ and the evidence of opportunistic infections in ATL patients⁴² all strengthened the notion that an HTLV-like virus was involved in AIDS. Sixth, although the AIDS disease was first recognized as an epidemic in the United States which was now spreading to Europe and Asia, the disease probably originated in Africa and Haiti³⁷ (Fig. 1), both endemic areas for the then known human T-lymphotropic retroviruses. Finally, once the first human retroviruses had been found, it was reasonable to expect that this class of viruses may be involved in other human diseases as well.

As the technology for sensitive assays for retroviruses and for culturing T lymphocytes *in vitro* was already available, it was a logical place to begin. In 1983, we found HTLV-I-related sequences from 2 of 33 AIDS patients⁴³ and rare virus isolates⁴⁴. We recognized that this could be caused by opportunistic infection with HTLV-I itself which was, nonetheless, important to document in this population. We also suggested that these viruses in AIDS patients could have been minor variants of HTLV-I and could have been involved in the cause of AIDS. Later, detailed analyses of these isolates showed they were HTLV-I itself and not minor variants⁴⁵. Therefore, we conclude that they are opportunistic infections.

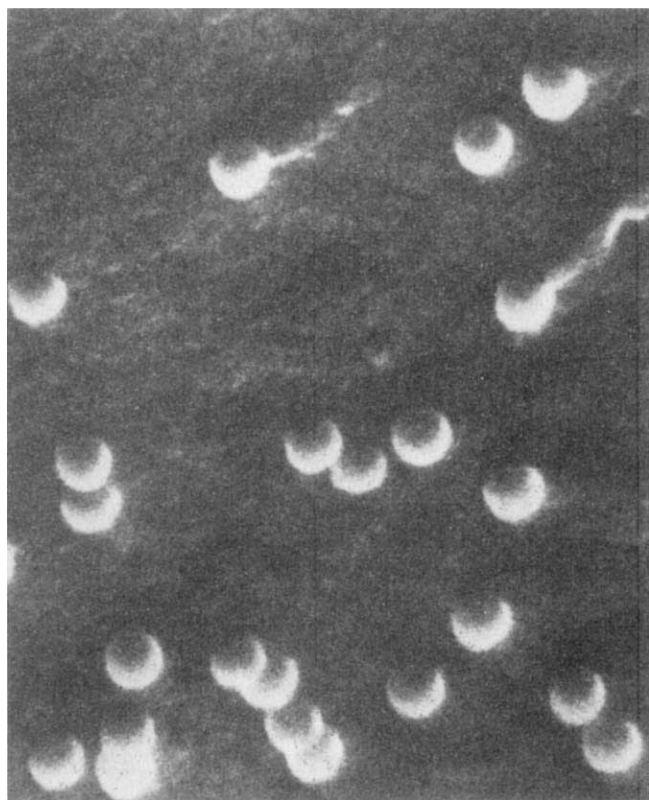


Fig. 2 Scanning electron micrograph ($\times 45,000$) of budding and absorbed HTLV-III on the surface of an H9 cell. (Courtesy of M. A. Gonda and K. Nagashima, Program Resources, Inc.)

Using the same approaches for the isolation of HTLV-I and HTLV-II, we also detected transient reverse transcriptase activity and retrovirus-like particles by electron microscopy from cultured T cells of peripheral blood and lymph node of a few AIDS and 'pre-AIDS' patients, which were apparently different from HTLV-I or HTLV-II. However, unlike previous experiences, we could not maintain growth of the virus or the T cells in culture. Therefore, we could not characterize them because of lack of reagents. In 1983, Barré-Sinoussi *et al.* using a modification (addition of anti α -interferon) of the same cell culture protocol (lectin-stimulated T cells grown with IL-2) used for the isolation of HTLV-I detected a retrovirus in T cells of a lymphadenopathy patient⁴⁶. They recognized this virus to be a human T-lymphotropic virus. Subsequently, they named this virus LAV or IDAV (for immunodeficiency associated virus). The lack of specific reagents at that time precluded the conclusion that samples from different AIDS patients contained the same virus and a clear link to AIDS could not be established. Nonetheless, this report was important as being the first description of the detection of the virus which would be defined as the cause of AIDS. The problems of large-scale virus production, development of specific viral reagents and linkage of the virus to the cause of AIDS were eventually solved once we recognized the cytopathic effects of the virus in culture (see below).

Popovic *et al.* developed clones of a permanently growing T4 cell line susceptible to virus infection but at least partially resistant to its cytopathic effect providing the first successful transmission of the virus to a permanently growing cell line⁴⁷. Highly resistant cell clones (H9 or H4) of a leukaemic T-cell line were chosen as targets for virus isolation and detection. Figure 2 depicts virus particles budding from or absorbed to an H9 cell. We initially described 48 isolates⁴⁸ but now have over 100 (ref. 49). By using similar target cell lines or rationale, viruses have now been isolated from AIDS patients in other laboratories⁵⁰. The availability of continuous, high-titre producer cell lines also allowed development of purified viral

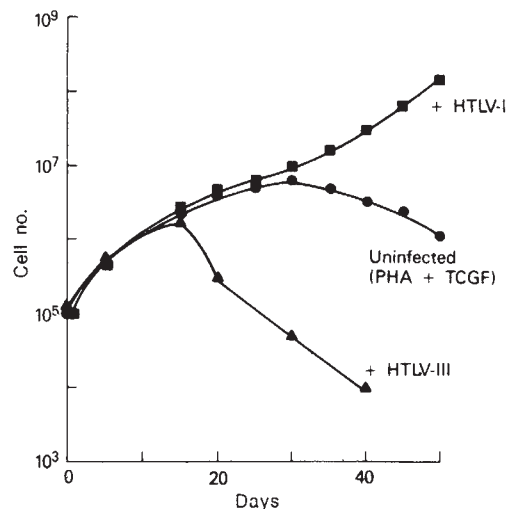


Fig. 3 Biological effects of HTLV-I and HTLV-III on normal cord blood T lymphocytes. Cumulative cell numbers were recorded after different days of infection. ●, Normal, uninfected, mitogen-stimulated T cells derived from the umbilical cord blood grown in the presence of 10% TCGF in long-term cultures. ■, HTLV-I-infected cord blood T cells cultured under the same *in vitro* conditions as uninfected cells. ▲, HTLV-III-infected cord blood T cells cultured under the same conditions.

reagents for extensive seroepidemiological studies (see, for example, 51, 52) and for comparing the 48 different initial isolates. These results showed that each of these isolates belonged to the same virus group. The fact that these viruses were all found in people from risk groups or from patients with AIDS, coupled with seroepidemiological data showing a high percentage of seropositivity in the same groups of individuals but not unaffected and non-risk people, allowed us to link the virus to the cause of AIDS^{47,48,51}. The first serological evidence of a human retrovirus involvement in AIDS was obtained by M. Essex and co-workers using HTLV-I reagents^{53,54}. These studies clearly identified antibodies in sera of AIDS patients and asymptomatic individuals which were the source of infection of blood transfusion recipients. These findings may now be explained by cross-reactivities of antigens of HTLV-I with those of the AIDS agent⁵⁵. Because of their tropism for the OKT4⁺ T lymphocyte and some biological, biochemical and structural features in common between the new virus and HTLV-I and HTLV-II, we adopted the nomenclature convention for human retroviruses agreed on by US, Japanese and European colleagues at the Cold Spring Harbor HTLV Symposium in 1983, and named this virus HTLV-III. Just as different isolates of papilloma viruses and hepatitis viruses may not share much homology, the term HTLV-III does not depend on an arbitrary extent of nucleotide sequence homology to HTLV-I and HTLV-II. Subsequent studies demonstrated that these viruses were closely related to the LAV isolate.

The cytopathic activity *in vitro*, the repeated isolation from patients with AIDS and people at risk, and results of the seroepidemiological studies are all consistent with HTLV-III being the aetiological agent of AIDS. However, HTLV-III is only indirectly involved in the lymphadenopathy syndrome (LAS) or AIDS-associated neoplasms such as Kaposi's sarcoma and B-cell lymphoma, as viral genetic information is only found in a small fraction of cells in lymph nodes of LAS patients and not detectable in Kaposi tumour⁵⁶ or B-cell lymphoma tissues (our unpublished observations). The association of Kaposi's sarcoma with AIDS deserves special mention. This otherwise extremely rare malignancy occurs predominantly in a restricted risk group, that is, the homosexuals, and can occur in the absence of any apparent T-cell defect in the patients. Therefore, the precise role of HTLV-III in the predisposition of the disease is unclear but merits intensive study. Virus has been obtained from blood, bone marrow, lymph nodes, spleen, plasma⁴⁸⁻⁵¹,

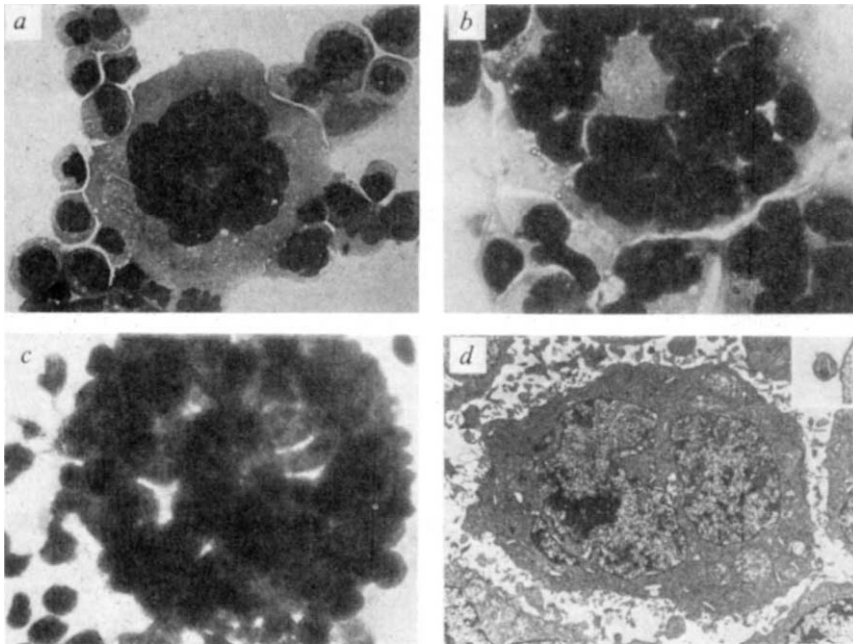


Fig. 4 Syncytia formation of cells infected by HTLV-I, -II and -III. A common feature of HTLV cells is the development of multinucleated cells and cells with multilobulated nuclei. These aberrant cells may be normal in size or in the form of giant cells, commonly called syncytia. *a*, Cell culture of human umbilical cord blood leukocyte transformed by HTLV-I, showing both the normal-sized cells with multilobed nuclei and a dramatic giant cell; *b*, large multinucleated cell from the peripheral blood leukocyte transformed by HTLV-II; *c*, giant multinucleated cell infected by HTLV-III; *d*, electron micrograph of a similar giant syncytial cell showing virus production from its plasma membrane. Inset shows a mature particle of HTLV-III. (Courtesy of Z. Salahuddin). *a-c*, $\times 1,000$; *d*, $\times 7,500$; inset $\times 30,000$.

semen^{57,58} and saliva⁵⁹ of infected individuals. These results not only support previous documentations of transmission of AIDS by blood products (for example, factor VIII) and sexual contact, but also suggest that salivary exchange is an infrequent mode of transmission.

Transformation and cytopathic effects

One of the most distinctive properties of the HTLVs is their ability to reproduce *in vitro* some of their biological properties *in vivo*. These properties include immortalization and transformation of normal T lymphocytes by HTLV-I and HTLV-II⁶⁰⁻⁶² and cell killing by HTLV-III⁴⁷. Normal lymphocytes obtained from cord blood, bone marrow or peripheral blood grow transiently in the presence of IL-2 after activation with phytohaemagglutinin, which induces the synthesis of IL-2 receptors⁶³⁻⁶⁵ (Fig. 3). However, they usually enter a 'crisis' period between 30 and 40 days when growth declines or terminates. So far, no permanent cell lines have been established from normal, uninfected T-lymphocytes. In contrast, when these T cells are infected with HTLV-I or HTLV-II, some of the cells grow indefinitely. A remarkable and consistent difference between the HTLV-I-positive ATL cells and normal T4 cells is the constitutive expression of IL-2 receptors by ATL cells^{64,66,67} (see ref. 68 for review). Moreover, the IL-2 receptors of these virus-positive leukaemic cells cannot be down-regulated⁶⁹ and are unusually abundant⁶⁸⁻⁷⁰. Like the primary ATL cells, the T-cell lines immortalized by *in vitro* infection with HTLV-I or -II are: predominantly OKT4⁺ (although occasional OKT8⁺ cell lines have also been established); have partial or complete independence of exogenous IL-2; express high levels of IL-2 receptors, transferrin receptor and HLA-DR; and often display lobulated nuclei and multinucleated giant cells (Fig. 4)^{60,61}. Most HTLV-I or -II infected T cells do not become transformed, but infection may seriously impair their immunological function³⁹⁻⁴¹. This may explain the observation that the OKT4⁺ ATL cells obtained directly from patients do not have helper activity⁷¹ and may also explain why HTLV-I-infected individuals are prone to opportunistic infections⁴². Furthermore, HTLV-I-infected T4 cells may cause polyclonal B-cell activation³⁹ and selective killing of certain cytotoxic T cells^{41,72}. All these properties of HTLV-I are limited manifestations of the more profound immunosuppressive and cytopathic properties of HTLV-III.

Normal T cells infected *in vitro* with HTLV-III initially resemble the HTLV-I- and HTLV-II-infected cells in the appearance of multinucleated giant cells (Fig. 4), but instead of infinite

growth of some cells, a dramatic loss of cell viability is noted within 2-3 weeks (Fig. 3). The onset of this premature cell death correlates with expression of virus. Both virus infection and cell killing occur specifically in the OKT4⁺ cells which become depleted along with a decline and eventual cessation of virus production. These may be the only type of T cells infected by HTLV-III because an epitope of the T4 antigen was recently shown to be a component of the receptor for HTLV-III^{73,74}. The specific infection and depletion of the OKT4⁺ helper T lymphocytes *in vitro* by HTLV-III is again a mimicry of the clinical manifestation of the AIDS disease analogous to the infection of the same cell by HTLV-I, leading to *in vitro* immortalized growth and *in vivo* T-cell leukaemia.

T cells are not the only cells that can be infected by HTLV-I, -II and -III. Subpopulations of B cells were infected by HTLV-I^{75,76} and by HTLV-III^{77,78}. The latter seem to be B cells that express a T4 epitope⁷³. Furthermore, fibroblasts⁷⁹ and vascular endothelial cells⁸⁰ could also be infected with HTLV-I under special laboratory conditions. Cells in the brain of AIDS patients which are not lymphocytes have also been found to be infected by HTLV-III⁸¹, although the exact nature of these cells is not yet determined. Recently, infection of macrophages by HTLV-III was demonstrated (Z. Salahuddin and R. C. Gallo, unpublished; R. A. Weiss, personal communication). Although infection of non-T cells by retroviruses of the HTLV family is not yet known to have relevance to their primary pathogenic effects, the infection of brain cells is obviously pertinent to the dementia and other neurological abnormalities associated with HTLV-III infection, and infection of any of these cells could account for possible virus reservoirs.

Genetic structures of the HTLV genomes

Molecular clones of the genomes of all three subgroups of HTLV have been obtained⁸²⁻⁸⁸ and the complete nucleotide sequence of one or more isolates of each subgroup has been published⁸⁹⁻⁹⁵. Figure 5 depicts the organization of potential coding regions of these viruses compared with two prototype mammalian and avian type-C retroviruses (Moloney murine leukaemia virus, M-MuLV and Rous sarcoma virus, RSV), and with BLV (distantly related to HTLV). The genomic structure of STLV-I closely resembles that of HTLV-I.

In addition to several general biological features common to HTLV-I and -III, there are some striking structural features that are common among the HTLVs and the related BLV. First, the notable absence of the small phosphoprotein (prol-gagtin) interposed between the amino-terminal gag protein (myrgagtin) and

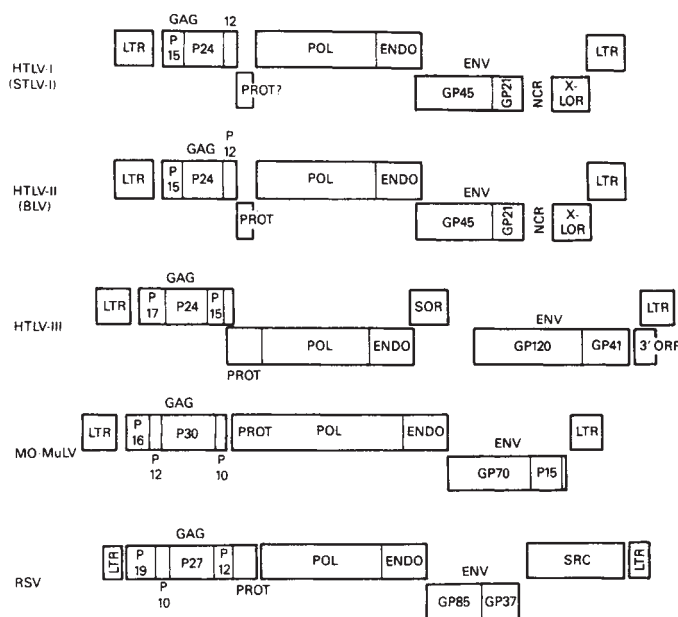


Fig. 5 Genetic structures of retroviruses. ENDO, endonuclease; PROT, protease; NCR, noncoding region; X-LOR, long open reading frame in pX; SOR, short open reading frame; 3' ORF, 3' open reading frame.

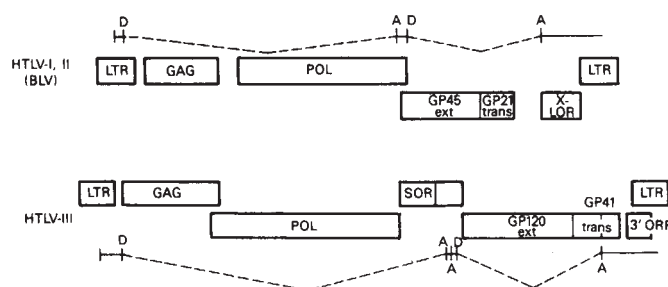


Fig. 6 Generation of multiply spliced 2.0-kb mRNA of HTLV-I, HTLV-II, BLV and HTLV-III. D and A denote donor and acceptor splice sites.

gene. However, the 3' *orf* gene is polymorphic in that some HTLV-III proviruses contain one or more termination codons so that its coding capacity ranges from 134 to 236 amino acids⁹². A biologically active provirus¹⁰⁵ clone has the truncated form¹⁰⁶, but the single stop codon may be suppressed. In addition, several short ORFs, that encode proteins 6–10K in size can be found. Two or more species of abundant mRNA ~2 kb in size are found in all HTLV-III-infected cells¹⁰⁶. Analyses of complementary DNA clones derived from these mRNAs showed a doubly spliced structure indicated in Fig. 6 (refs 95, 107). The first donor site is located in the non-translated leader sequences of the *gag* gene. The short middle exon is derived from sequences following *sor*, and uses one of two possible acceptor sites. The last exon contains 414 nucleotides of the *env* gene and the rest of the 3' end of the genome. For the messenger RNA with the shorter middle exon, the only reasonably long open reading frame with a methionine codon is 3' *orf*. Therefore, it probably encodes the 3' *orf* gene product. The mRNA species with a longer middle exon contains additional short open reading frames that can encode protein(s) of 6–10K in size. Even more complex splice patterns involving additional short exons in the *pol* and *sor* regions have been observed⁹⁵. In any case, the generation of a 2.0-kb mRNA by multiple splice events is reminiscent of the *x-lor* mRNA of HTLV-I, HTLV-II and BLV.

The *env* gene of HTLV-III differs from that of most other known retroviruses. The major exterior glycoprotein is large and heavily glycosylated, with >20 potential glycosylation sites. The carboxy-terminal *env* protein has a region that closely resembles the transmembrane protein of other retroviruses. However, there is an additional domain at the carboxy-terminus that is hydrophilic and may be intracellular.

HTLV-III has many parallels with the ungulate lenti-retroviruses, particularly visna virus. These include cytopathic effects *in vitro*, accumulation of a large amount of unintegrated viral DNA in the infected cells^{56,86} (a property shared by other cytopathic retroviruses), the capacity to infect cells in the brain⁸¹, viral morphology and distant nucleotide sequence homology¹⁰⁸. Recent studies have shown that HTLV-III also shares distant sequence homology with caprine arthritis-encephalitis virus and equine infectious anaemia virus¹⁰⁸. Therefore, HTLV-III and the ungulate lenti-retroviruses have diverged from a common progenitor and HTLV-III may be a human lentivirus.

Transactivation and retrovirus classification

HTLV-I, HTLV-II and BLV differ from other leukaemia and sarcoma retroviruses in that they do not contain cell-derived oncogenes and require neither active viraemia nor a conserved site of provirus integration for transformation/leukaemogenesis (see ref. 36 for review). The latter observation suggests that a *trans*-acting viral element must mediate their biological activity. Several lines of evidence indicate that the *x-lor* gene is critical for transformation: its high degree of conservation, its retention and expression in almost all transformed cells, including those containing single proviruses deleted in the entire *env* gene¹⁰³, and the fact that it is unrelated to all previously known retrovirus-replicative genes. *x-lor* is more similar to the transforming genes

the major capsid protein (largagtin) (see ref. 96 for terminology). This immediate juxtaposition of largagtin to myrgagtin has not been found in any other retroviruses. Second, although the HTLVs all lack cell-derived oncogenes, they accommodate one or more coding regions in addition to the usual viral replicative genes (*gag*, *pol*, *env*). The relatively small size of the largagtin (p24) and the glycosylation of the transmembrane protein (gp21 or gp41) are additional features that distinguish them from the mammalian type-C retroviruses.

HTLV-I and HTLV-II have very similar organization^{88,90}. One apparent difference is the presence of a noncoding region between the *gag* and *pol* genes in HTLV-I and a protease gene in an analogous position in HTLV-II. Although an HTLV-I variant that has been sequenced also has a noncoding region⁹⁷, neither of these proviruses has been shown to be biologically active. Therefore, it is still possible that the lack of a protease gene signifies a defect in these particular proviruses and not a real difference between HTLV-I and HTLV-II. BLV also has a protease gene at the same place⁹⁸. The gene, designated *x-lor*, has a subdomain at the 3' end of the pX region first identified by Seiki *et al.*⁸⁹. This gene is highly conserved between HTLV-I and HTLV-II⁹⁰ and encodes protein products of relative molecular mass 42,000 (42K) and 38K respectively^{99,102}. A 2.0-kilobase (kb) *x-lor* messenger RNA is apparently generated by a complex splicing mechanism^{103–105} (Fig. 5). Three exons are joined by using a donor splice site located in the R region of the LTR, an acceptor upstream of the *env* gene followed by a donor 190 nucleotides apart and a second acceptor splice site that also marks the beginning of *x-lor*. The middle short exon contributes an initiator ATG codon, which is also the initiator codon for the *env* gene, plus a G residue which puts the rest of *x-lor* in the correct reading frame. This double-splice mechanism is unprecedented in mRNA transcription of other retroviruses.

The genome of HTLV-III has a few peculiar features^{92–95}. It uses a primer binding site homologous with lysyl transfer RNA. It has at least two relatively short open reading frames (ORFs) of unknown function: *sor* (also called orf-1, Q or P'), located between *pol* and *env*, and 3' *orf* (also called orf-2, F or E'), after *env* and extending into U3. Peptides derived from 3' *orf* sequences are immunogenic against the sera of AIDS patients (G. Franchini *et al.*, unpublished), suggesting that it is a functional

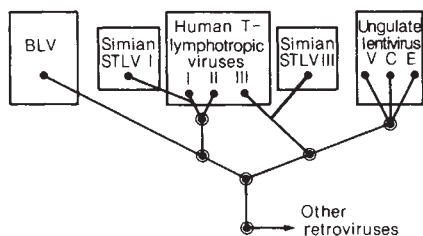


Fig. 7 The *trans*-activating retroviruses. STLV-I and STLV-III: simian T-lymphotropic virus types I and III. V, visna virus; E, equine infectious anaemia virus; C, caprine arthritis and encephalitis virus.

of DNA tumour viruses both in its lack of a close cellular counterpart and its action as a transcriptional activator. Sodroski *et al.*¹⁰⁹ first described the phenomenon that the steady-state mRNA level, and consequently the enzymatic activity, of a bacterial gene (*CAT*) linked to the viral LTR is greatly enhanced in virus infected cells expressing *x-lor*. They called this *trans*-acting transcriptional (TAT) regulation. Recent evidence for a direct role of *x-lor* in TAT was provided by the demonstration of *transcriptional* activation of an LTR-linked *CAT* gene in cells transfected with only the *x-lor* gene¹¹⁰. As *x-lor* now has a defined function, we, in agreement with Sodroski and colleagues¹¹¹, have renamed it the *tat* gene. It has been speculated that, like some of the DNA virus proteins¹¹²⁻¹¹⁴ (for example, E1a of adenovirus), *tat* not only activates transcription of viral genes, but also positively or negatively regulates transcription of some cellular genes; these would be genes involved in lymphoid cell proliferation. Activation of these genes on virus infection would then lead to immortalization and subsequently transformation of these cells. As shown in Fig. 7, this mechanism of leukaemogenesis differs from those of the acute or chronic leukaemia viruses.

HTLV-III exhibits TAT activation¹¹⁵ that is one or two orders of magnitude greater than that of HTLV-I¹⁰⁹, HTLV-II¹⁰⁹ and BLV¹¹⁶. As described above, there are several potential extra coding regions that could accommodate this function. Recently, the *tat* gene was mapped to a region previously thought to be non-coding, immediately before the *env* gene^{108,117}. The mRNA encoding the *tat* protein is one of the doubly spliced 2-kb mRNAs¹⁰⁶. This is a remarkable coincidence with the doubly spliced 2-kb mRNA of the *tat* gene of HTLV-I, -II and BLV. It is not known whether TAT is an integral part of the cytopathic activity of the virus. We have obtained a molecular clone of HTLV-III which directs the production of virus particles that are infectious¹⁰⁵, cytopathic for T4 cells¹⁰⁵ and capable of mediating *trans*-activation (our unpublished data) on transfection into cells. It would be of interest to correlate the biological functions with specific mutations in this virus genome in further transfection studies.

Members of the ungulate lentivirus group also exhibit TAT activation¹¹⁸. Thus, HTLV-I, HTLV-II, HTLV-III, STLV-I, BLV and the ungulate lentiviruses not only share a varying degree of homology and/or several biological and structural features, but they also have a viral gene which mediates TAT activation. We suspect that STLV-III will also have this property and we propose a tentative family tree of this group of viruses (Fig. 8).

Genomic diversity of HTLV-III

We first discovered the heterogeneity of different HTLV-III isolates when we analysed a few of our isolates by Southern hybridization⁵⁶. Now, with >30 of >100 isolates in our laboratory analysed, >10 cloned and partially or completely sequenced, the picture that emerges is that HTLV-III constitutes a continuous spectrum of closely related to more divergent viruses¹¹⁹. LAV, the original isolate of the Pasteur group⁴⁶, and ARV, an isolate obtained more recently⁵⁰, both fit within this spectrum. The extent of divergence can vary from 1-2% (LAV, HTLV-III_{BH10}; HTLV-III_{MN}, HTLV-III_{SL}) to 7-8% (ARV and

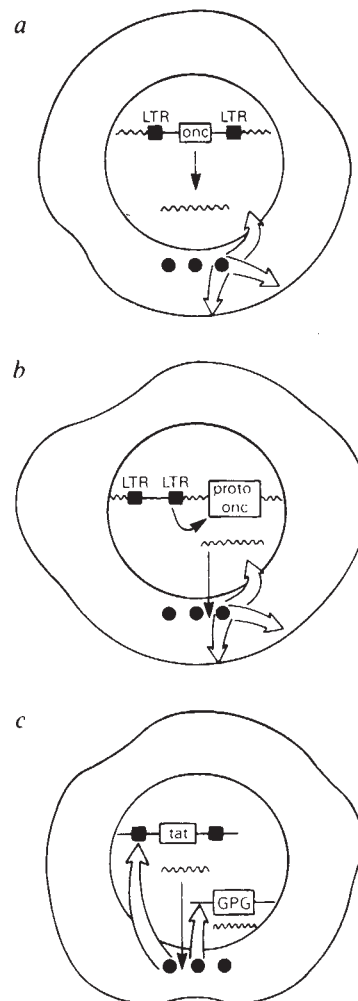
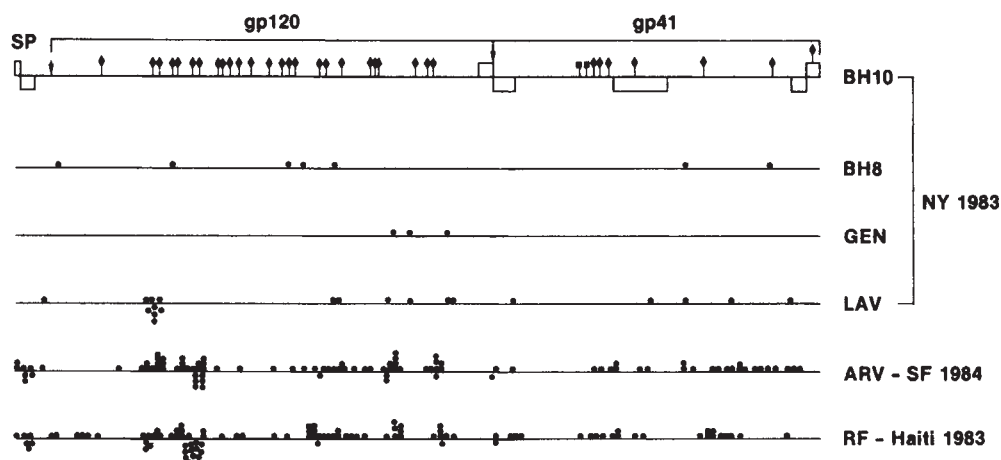


Fig. 8 Mechanisms of leukaemogenesis. *a*, Acute leukaemia viruses; *b*, chronic leukaemia viruses; *c*, HTLV/STLV/BLV. *a*, Acute leukaemia viruses have captured cell-derived genes (*onc* genes) which encode transformation-specific proteins¹⁴¹. Some of these proteins are growth promoting proteins and may be localized to the membrane (for example, growth factor receptors), cytoplasm (for example, growth factors) or nucleus (for example, DNA binding proteins). These possible sites of action are shown by arrows. Because these viral proteins are *trans*-acting, no conserved site of provirus integration is necessary. *b*, Chronic leukaemia viruses activate cellular genes by proximal integration of the provirus¹⁴². The activated cellular genes may be proto-*onc* genes. The mechanism of *cis* activation requires conserved site(s) of integration. *c*, The HTLV/STLV/BLV family of viruses make a nuclear protein (TAT) which activates transcription in *trans*. Therefore, a conserved integration site for the provirus is not necessary. It is speculated that the *tat* gene product will activate the expression of growth promoting genes (GPG) specific for lymphoid cells.

HTLV-III_{RF} vs. LAV)¹²¹. It is of interest and disconcerting that the most divergent part of the genomes lies within the major exterior glycoprotein portion of the *env* gene, as epitopes involved in virus neutralization generally reside in this region. Comparing the three most divergent known isolates, namely, the BH 10 and RF strains of HTLV-III and ARV, which differ in 10-20% of amino acids from each other in the *env* sequences, one can locate 'hot spots' of mutation (Fig. 9) (our unpublished data). These hot spots may correspond to dispensable regions in the envelope which can allow a degree of freedom to change, or to epitopes that drift with immunoselection. On the other hand, there are also regions of extreme conservation which probably represent critical functional domains, for example, that which interacts with the cellular receptor. The most obvious conserved sequences are those flanking the cleavage site for the

Fig. 9 Divergence of the *env* gene of HTLV-III. ♦, Glycosylation sites; ■, hydrophilic stretches; ▒, hydrophobic stretches; □, placement of cysteine residue; ●, non-conservative amino-acid change from the prototype BH10. Changes involving amino acids having similar side chains are considered conservative.



transmembrane protein. These may be exploited for development of diagnostic or therapeutic reagents that will be broadly cross-reactive.

Public health, social and ethical issues

The public health implications of human retrovirus infections are only now beginning to be appreciated. Though less in the public eye than HTLV-III and AIDS, HTLV-I has been documented to be a major leukaemogen in endemic areas where it accounts for 50–70% of all lymphoid leukaemias of adulthood. HTLV-II, which was first obtained from a patient with hairy cell leukaemia¹²⁰, is relatively rare. Recent reports from the United Kingdom and the United States indicate that certain populations of intravenous drug users have exceptionally high rates of HTLV-I (and HTLV-II) infection¹²¹. In Japan, blood transfusion has been implicated as a major mode for HTLV-I transmission in that population¹²². The potential thus exists that a potent human leukaemogen is being introduced into previously uninfected populations. Because of the relatively low disease-to-infection ratio and long latency of this virus, the impact of these findings is not yet felt, but an increase in T-cell malignancies caused by this virus is expected.

The medical and social problems associated with HTLV-III (LAV) and AIDS have created unprecedented problems for modern medical and public health researchers. Compared with the normal rate of progress in research on human diseases, AIDS and HTLV-III (LAV) basic science research is very fast¹²³, caused by earlier experiences with animal retroviruses and with HTLV-I and -II and the great progress made in fundamental molecular biology and immunology in the previous decade. However, despite rapid progress, disease development continues to outrun the science. AIDS is emerging as the first major lethal pandemic of the second half of the twentieth century.

As shown in Fig. 10, there is an acceleration in the incidence of AIDS in all risk groups and recent estimates suggest 40,000 new cases of AIDS in the United States in the next 2 years. The direct lifetime hospital costs for care of such patients is calculated to be \$42,000 per patient. Thus patient health care costs for this epidemic over the next 2 years in the United States alone may exceed 1,000 million dollars¹²⁴.

Estimates of the number of people already infected by HTLV-III have been conservatively placed at between 400,000 and 1,000,000, but this may be a significant underestimate, given emerging data concerning unexpectedly high rates of seropositivity in some risk groups as well as the findings of some virus-positive, seronegative individuals^{125,126}. Preliminary estimates demonstrate that HTLV-III seropositivity translates into AIDS disease in up to 8–10% per year with an equal or higher rate of AIDS-related conditions also developing in a short period. These include thrombocytopenia, lymphadenopathy syndrome, neurological manifestation, and diverse malignancies, particularly Kaposi sarcoma, B-cell lymphomas and some

carcinomas¹²⁷. In addition, we must consider the possible long-term sequelae, such as chronic degenerative diseases, in monitoring HTLV-III-infected people in the coming decade.

The syndrome is also associated with profound psychological, social, economic and political concerns. Scientists can help to alleviate these problems. First, accurate information in reviewed scientific literature can be provided. Second, the blood-bank test for antibodies to HTLV-III can be implemented and some misconceptions clarified. For example, it has been said that seropositivity may not mean virus positivity. Yet, our co-workers can isolate HTLV-III from peripheral blood of >80% of people with serum antibodies to the virus. Because of the technical difficulties with some blood samples this value may approach 100%. Therefore, a properly performed positive result means the person is infected, and until proven otherwise the individual must be considered infectious. The opposite concern, that is, there may be false negatives, is valid, based on recent evidence for virus-positive antibody-negative homosexuals¹²⁶, but they represent less than 20% of virus-positive people. Clearly, we should proceed with blood-bank antibody tests and develop

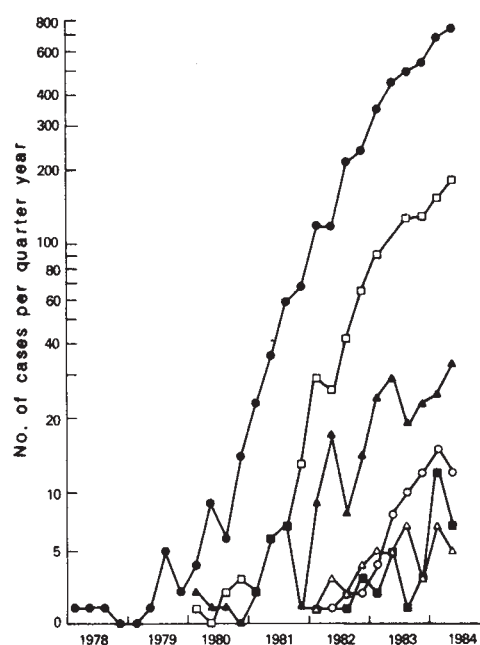


Fig. 10 Semilogarithmic graph of the number of new US AIDS cases diagnosed in each quarter year, 1978 to July 1 1984 (as of October 29 1984) by six hierarchical high-incidence groups¹⁴³. ●, Homosexual males; □, parental drug users; ▲, Haitians; ○, transfusion recipients; ■, haemophiliacs; △, heterosexual contacts. (Courtesy of J. Goedert and W. Blattner).

additional (for example, antigen) assays. A final, often expressed, reservation about a blood-bank assay is the possibility of motivating people from risk groups to donate blood so that they could learn the result. This problem can be solved by establishing centres for diagnostic testing. Although a precise test for serum antibodies to HTLV-III was available in our laboratory by December 1983, virus-positive blood continues to enter new populations and new countries. Thus, the application of technological advances is too slow. Removal of antibody-positive blood and the development of sensitive antigen assays for antibody-negative blood are tests that can now be performed.

Social questions arose from findings of HTLV-III in saliva⁵⁹ suggesting possible transmission by casual contact. The care of patients with AIDS has sometimes been refused by health-care workers. However, epidemiological studies indicate that saliva is not a major route of transmission, for example, non-sexual household contacts are generally virus-negative. In our experience, patients with AIDS have less virus in their blood than 'healthy' carriers or people with AIDS-related complex, and virus was isolated from saliva of healthy carriers, not from people with AIDS⁵⁹. We have tested many individuals who work with HTLV-III from many laboratories including our own and have never found an antibody-positive serum. All available evidence indicates that none of the human retroviruses are transmitted by inhalation. However, a recent report of sera conversion after a needlestick¹²⁸ shows the potential risk to health-care workers exposed to infected secretions and blood/blood products. Logically, then, the most useful safety precaution for HTLV-I, -II or -III is to avoid exposure to needles and to use disposable glassware. The greatest hazard is the handling of blood from a normal virus-positive individual by an unsuspecting and inadequately trained hospital employee.

Why is the virus widespread in homosexuals? The available evidence suggests that this is not caused by unusual susceptibility of this group *per se* but rather to the frequency of sexual contacts and the probability that in the United States and Europe HTLV-III first entered this group. In fact, heterosexuals are the major infected group in Africa¹²⁹, and studies in the United States show recent infection of heterosexuals^{130,131}. Therefore, transmission by several sexual routes as well as by blood or blood-products inoculation is likely. However, the risks of heterosexual transmission cannot yet be assessed and the future rates of infection of the heterosexual population for most parts of the world cannot be predicted. What is clear is that in most areas diseases caused by HTLV-III are new. Like HTLV-I, the AIDS virus probably originated from Africa, but unlike HTLV-I, it was until recently much more restricted to certain regions of central Africa^{132-134,145}, spreading to other regions of Africa, then to the United States and Europe, and now to other parts of the world (Fig. 1).

Conclusions

It is not purely coincidental that all human retroviruses isolated to date are tropic for mature T cells, as the discovery of T-cell growth factor or IL-2 has availed scientists of selective long-term cultures of these cell types. However, it is striking that the selection is specifically for the T4 subset. It is also striking that viruses closely related to these human retroviruses naturally infect multiple primate species, an unprecedented situation among retroviruses. In addition, all human T-lymphotropic viruses share many structural and functional features, some of which are unique. A list of the similarities between HTLV-III and HTLV-I (in most instances also HTLV-II) is presented in Table 1. In addition to those mentioned in the introduction, more salient features are: (1) transcriptional regulation (TAT) of viral and possibly cellular genes; (2) the presence of one or more extra non-cell-derived gene(s); (3) a novel (for retroviruses) double-splicing mechanism for generation of a 2-kb mRNA near the 3' end of the viral genome. The TAT phenomenon may be a key molecular mechanism involved in the abnormal cellular response (transformation/cytopathicity) to these viruses.

Table 1 Similarities between HTLV-III and HTLV-I (-II)

- *1 Human, exogenous, infectious retroviruses
- 2 Lymphotropism
- *3 Particular T4 tropism
- 4 Induction of syncytial formation *in vitro*
- 5 Impairment of T-cell function *in vitro*
- 6 Immunosuppression *in vivo*
- *7 Presence of highly related viruses (STLV-I, STLV-III) in Old World monkeys
- 8 Probable African origin
- 9 Mode of transmission: sexual (semen), blood, congenital
- 10 Indirect association with B-cell leukaemia/lymphoma
- 11 Size of major core protein (p24/25)
- *12 Lack of small phosphoprotein interposed between NH₂-terminal gag protein and p24
- *13 Common p24/25 epitopes with both heterologous (rabbit) antisera and a human monoclonal antibody
- 14 Stretch of nucleotide sequence homology in gag-pol region and LTRs
- *15 Double splice to generate 3' mRNA of ~2 kb.
- *16 Presence of non-cell derived gene(s) in addition to gag, pol and env
- *17 Trans-acting transcriptional activation of viral LTR

* Possibly unique to these viruses.

What is the abnormal cellular response to these events? *In vitro* models of T-cell transformation (HTLV-I and HTLV-II) and T-cell killing (HTLV-III) have been developed which have already provided some clues. For instance, in comparison with normal T cells there are two consistent features of HTLV-I and HTLV-II *in vitro*-transformed T cells and the HTLV-I-positive fresh leukaemic blood primary cells. (1) They constitutively express T-cell growth factor receptors⁶⁶⁻⁶⁸ and (2) class II (HLA-D/DR or Ia) antigens of the major histocompatibility complex, including self-Ia determinants, stimulate proliferation of the HTLV-I-infected T cells indiscriminately⁴⁰. Thus, autostimulation by a self-Ia determinant could promote clonal expansion of infected T cells. In the case of HTLV-III, infection is followed by persistence of unintegrated and integrated DNA⁵⁶. Cytoplasmic degeneration is seen following stimulation of the infected cells with antigen or lectin, associated with expression of various lymphokines and of the viral genes. Elucidation of the precise role of viral and cellular factors in these processes, and of the subsequent cascade of events leading to full manifestation of diseases, is being investigated by many laboratories.

Two of the three human T-lymphotropic viruses have been linked to human diseases: HTLV-I with ATL and HTLV-III with AIDS. ATL is found only sporadically in the United States and Europe, but is endemic in many areas of the world. AIDS is now spreading through Europe and Asia. Knowledge of the agents in these diseases leads to strategies for prevention and intervention. Elucidation of the structures and functions of these viruses, coupled with the power of molecular biology and recombinant DNA technology, allow a direct attack on the aetiological agent. Both HTLV-I and HTLV-III gag and env proteins expressed in bacteria have been shown to be useful diagnostic tools^{135,136} and will soon be available for blood-bank assays. A vaccine may be more appropriate for AIDS than ATL as the disease is more acute and widespread. Recent demonstrations of neutralizing antibodies in AIDS and pre-AIDS patients (refs 137, 138 and D. Ho and M. Hirsch, personal communication) are encouraging for the prospects of a vaccine, and large fragments of the env protein expressed in prokaryotes are ready for appropriate manipulations and injection into monkeys.

Furthermore, inhibitors of virus replication (for example, reverse transcriptase inhibitors) have already been used therapeutically¹³⁹, but it is still too early to draw conclusions on the efficacy of this approach. Attempts to kill the reservoir of infected cells by use of monoclonal antibodies against viral or virus-induced cellular markers are also in progress¹⁴⁰. It is to be hoped that a combination of all these approaches will curb the spread of these diseases.

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The magnetic reversal record is not periodic

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Statistical analysis of one version of the magnetic reversal record suggests that the frequency of reversals has changed cyclically with a period of 30 Myr starting at least 165 Myr ago. But factors other than periodicity affect the analysis used and here I show that there is no compelling evidence for periodicity in the reversal record. The results of essentially identical analyses that have been applied to other geolocal data should also be re-evaluated.

RAUP's analysis of the timing of reversals of the Earth's magnetic field¹ supports some earlier findings of periodicity of the order of 10^7 yr^{2,3}, although others found that the data were not inconsistent with random timing^{4,5}. Discrepancies among studies and the accuracy of any individual study could depend on differences among the several magnetic reversal data sets analysed⁶⁻⁹. However, I consider here the application of the methodology and not the differences among data sets. The reversal record of Harland *et al.*⁶ analysed by Raup is used throughout this study.

The method used by Raup¹ was developed independently by Raup and Sepkoski¹⁰ and Stothers¹¹. It has the advantages that it is not strongly sensitive to missing data and that confidence levels can be generated via a 'bootstrap' technique. The basic method has also been applied to the timing of biological extinctions^{10,12}, tectonic episodes¹³, sea-level changes¹³, certain types of igneous activity¹³, meteorite impacts¹³ and solar activity¹¹. Because geologists, biologists and astronomers all have an interest in these results it is appropriate to consider the validity of the statistical methods used and the conclusions which can be drawn from them.

In Raup's method the measure of periodicity is taken as the dispersion of observed events around the events predicted by a linear model, $t = t_0 + nP$, where t is the predicted age, t_0 is the age of the most recent predicted event, termed the phase, P is the time between predicted events, termed the period, and n is an integer ($n = 0, 1, 2, \dots$). A computer algorithm searches for the phase that minimizes dispersion for each period over a range of trial periods.

Circular model of periodicity

A conceptually more simple and computationally more efficient model is circular rather than linear. A time line can be 'wrapped' around a circle, the circumference of which represents a trial period. A series which is not periodic will tend to plot uniformly around the circle regardless of the trial period selected. A periodic series, however, will tend to form a cluster at one point on the circumference when the correct trial period is used. The angular location of the cluster relative to 0° (the present) gives the phase. The location of the cluster can be calculated analytically, thus eliminating a time-consuming search.

Ages of individual events, t_i , are converted into angles, a_i , for each trial period P :

$$a_i = (2\pi t_i / P) \bmod (2\pi)$$

where i ranges from 1 to N , the number of events. The phase which minimizes dispersion for a period is:

$$t_0 = (P/2\pi) \tan^{-1}(S/C)$$

where $S = (\sum \sin a_i) / N$ and $C = (\sum \cos a_i) / N$. A complete summary of the mathematics of circular transformations of linear data and circular statistics is given in ref. 14.

A residuals index¹¹ which is maximized when dispersion is minimized has been used as a measure of periodicity in the linear case. Peaks in a plot of residuals index against trial period (Fig. 2 in ref. 1) indicate periods for which there is some cluster-

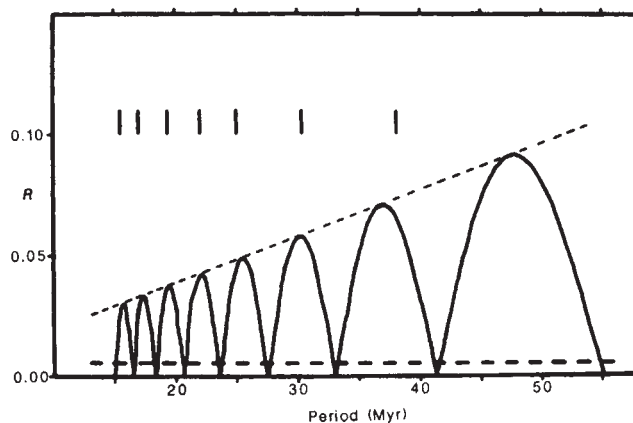


Fig. 1 Circular mean vector magnitude (R) plotted against trial period to show wrapping peaks. The data consist of 296 events spaced regularly at 0.56-Myr intervals, thus duplicating the number of events and the record length of the Harland *et al.* reversal series⁶. The amplitude of these peaks increases linearly with period. The locations of peaks determined by Raup¹ are shown as vertical bars. The correction for the wrapping effect described in the text reduces the R values for the pseudo-uniform data below the level of the horizontal dashed line.

ing of data. An analogous measure (the circular mean vector magnitude) and an analogous plot is used here in application to the circular model. The mean vector magnitude is calculated as:

$$R = (S^2 + C^2)^{1/2}$$

Raup¹ suggested that the statistical significance of a residuals peak can be estimated by comparing its amplitude with the distribution of peak amplitudes generated by analysis of Monte Carlo randomizations of the observed reversal record. His analysis yielded one highly significant peak with a period and phase (30 Myr starting 165 Myr ago) which was interpreted as a stationary periodicity in magnetic reversal frequency.

Straightforward application of the circular method to the reversal data of Harland *et al.*⁶ yields results virtually identical to those obtained by Raup¹ on the same data, suggesting that the two methods are truly analogous. Here, the application of such an analysis to the reversal data is reconsidered in terms of the accuracy of the results and the interpretation of results as evidence for a 30-Myr periodicity.

Record length effect

The accuracy of the analysis is affected by the finite length of the time series, an effect that can be deduced from the circular model. If the length of the series is an integral number of times the trial period, the series wraps around the circle so that all portions of the circumference are covered by the same number of wraps. However, in the general case some portion of the circumference is covered by one more wrap than the complemen-

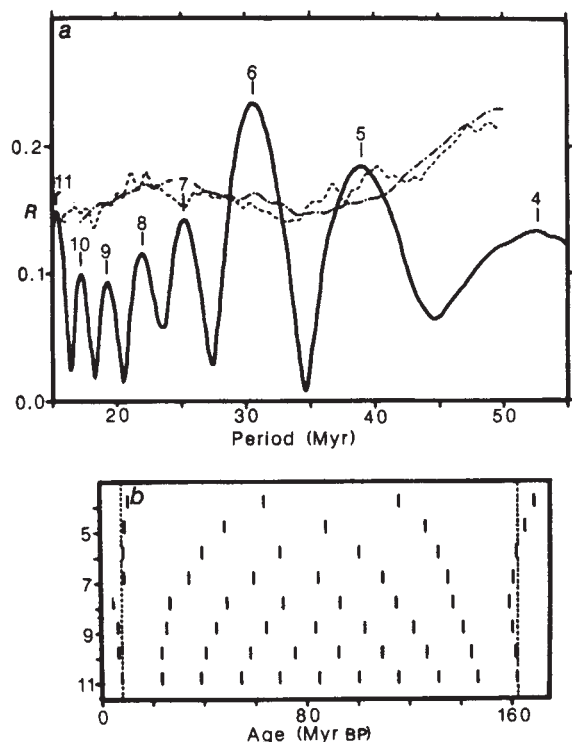


Fig. 2 *a*, Analysis of the Harland *et al.* reversal data⁶ using the wrapping correction. The peaks are numbered by the number of events predicted over the length of the data record; ---- and - · - · - show the 95% confidence level based on 100 and 400 randomizations, respectively, of the observed data. *b*, Ages of events predicted by each of the peaks shown in *a*. The vertical dashed lines show the mean ages of events which are predicted coherently by all of the peaks.

tary portion. For example, for a trial period of 30 Myr a 160-Myr series wraps five times around most of the circle but six times around a third of it. Therefore, in the general case the density of events along the circumference is not uniform even if the data are uniformly distributed in time.

The problem arises when many events occur per cycle of the hypothesized period and thus when there is a periodic variation in the frequency of events rather than in the occurrence of individual events. Clearly, the analysis of the reversal data is affected as the average interval between reversals is 0.6 Myr whereas the periodicity hypothesized is of the order of 30 Myr. The wrapping problem is most obvious in the context of the circular model but affects the linear analysis in a similar way. Previous work^{1,10-13} has apparently not dealt with this wrapping effect.

To demonstrate the wrapping effect, a time series was constructed that consisted of the same number of events as the reversal record of Harland *et al.*⁶ but distributed uniformly over the same time span. This series obviously does not contain a periodicity of ~30 Myr and can be referred to as pseudo-uniform on that scale. The circular mean vector magnitude (R) plotted against P (Fig. 1) shows a regular series of peaks that results entirely from wrapping. The wrapping peaks occur at periods for which the amount of extra wrap on the circle is slightly <0.5 times the circumference. Amplitudes close to zero occur when there is no extra wrap.

The locations of peaks given by Raup (Fig. 2 in ref. 1) are closely and consistently correlated with the wrapping peaks, as shown here in Fig. 1. However, the amplitudes of the peaks for the reversal data are 2–3 times larger than those of the wrapping peaks and do not increase linearly with period. Thus, the length of the reversal record does produce a wrapping effect, but this effect cannot entirely account for the size and distribution of the peaks for the reversal data.

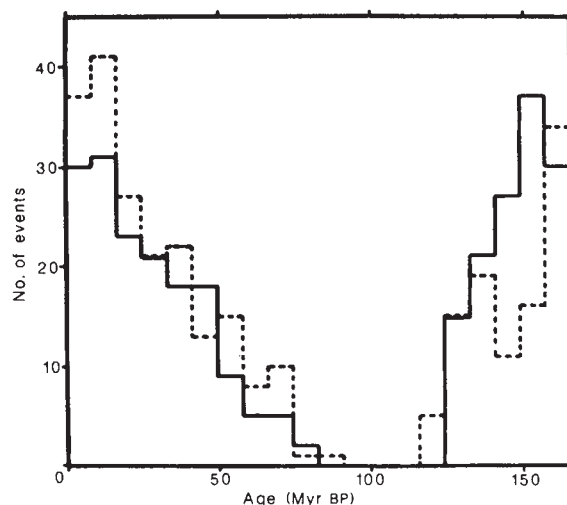


Fig. 3 Numbers of events per cell for the magnetic reversal data of Harland *et al.*⁶ (dashed line, 296 events) and one set of data simulating the long-term variation (solid line, 292 events). The events have been summed into 20 histogram cells, each with a width of 8.27 Myr, which span the reversal times series to illustrate the dominance of the long-term variation.

A correction for the wrapping effect involves weighting the data so that if there are m complete wraps and $m + 1$ extra wraps then all data which fall in the region of $m + 1$ wraps are given a weight of $m/(m + 1)$ relative to unit weight for the other data. This procedure reduces all variation for the pseudo-uniform data to a level below the horizontal dashed line in Fig. 1. The correction works well for pseudo-uniform data and as it is certainly an improvement over the uncorrected analysis it will be used in all subsequent analyses presented here.

The correction for wrapping does not have a strong effect on the locations or amplitudes of peaks for the reversal data (Fig. 2*a*). The 30-Myr peak is even more dominant than in the uncorrected analysis. Two lines representing the 95% confidence level were determined based on 100 randomizations (---, Fig. 2*a*) and on 400 randomizations (- · - · -) of the data using different sets of random numbers. Both lines have similar amplitudes and vary similarly as a function of trial period. There is no indication that more than 400 randomizations are required to form a good estimate of the 95% confidence level. Some real variation in this level clearly occurs, with higher values more typical for periods >40 Myr. Raup's¹ empirical procedure of using a single 95% value independent of period may be improved on; confidence levels can be calculated at each trial period.

Long-term variations

In contrast to the results without the correction, two peaks at 30.6 and 39.0 Myr exceed the 95% level (Fig. 2*a*). In one sense these peaks have statistical significance but their interpretation in terms of a stationary periodicity should not be accepted without considering the effects of long-term variations in the frequency of magnetic reversals. In addition to the hypothesized 30-Myr period in the reversal data there is an unambiguous variation in reversal frequency on the scale of 150 Myr (Fig. 3; see also Fig. 1 in ref. 1). Reversal frequency is very high near the present, peaking ~8 Myr ago and being high ~160 Myr ago, both times near the ends of the Harland *et al.* record⁶. McFadden and Merrill⁵ have suggested a continuous linear decrease in frequency from 160 Myr ago to the Cretaceous quiet period followed by a linear increase in frequency from the quiet period to the present.

The effect of such long-term variations in frequency on the periodicity analysis was tested by generating data that simulate the long-term variation without introducing any coherent short-

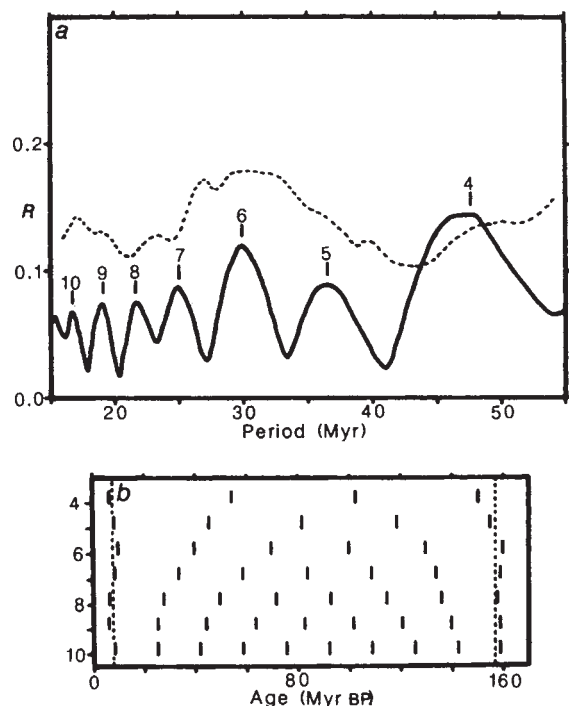


Fig. 4 *a*, Analysis of 292 events generated to simulate the long-term variation of the reversal data shown in Fig. 3. (See text for description of the simulation model.) The numbers above the peaks are the number of events predicted over the length of the data record. The dashed line shows the 95% confidence level based on 100 randomizations of the data. *b*, Ages of events predicted by the peaks shown in *a*. The vertical dashed lines indicate the mean ages of events predicted coherently by all the peaks.

term variation. Intervals between events were generated using γ variates with a shape parameter of 2 to reduce the numbers of unrealistically short intervals. The mean length of intervals was varied sinusoidally with frequency maxima modelled at 8 and 161 Myr ago. The γ density was adjusted to produce approximately the same number of events as the observed reversal record and to simulate the long-term variations in frequency (Fig. 3). Several sets of such simulated events were analysed with similar results. A typical example of such an analysis (Fig. 4*a*) has peaks nearly identical in location to those in the actual reversal analysis (Fig. 2*a*). One of the peaks (47 Myr) exceeds the 95% confidence level.

The locations of events predicted by the period and phase of each peak are shown in Fig. 4*b*. The ages of the predicted events are highly variable from one peak to the next, with the important exception that each peak predicts events close to 8 and 157 Myr. These times correspond closely to the times when the frequency of events is high, suggesting that the location of each peak reflects a harmonic of the long-term variation. An identical correspondence exists for the events predicted by peaks in the reversal record (Fig. 2*b*). Because the peaks for the simulated data correspond well with those for the reversal data, a test should be made to determine whether the 30- and 39-Myr peaks, as well as the others, are harmonic peaks unrelated to periodicity.

The period and phase of harmonic peaks should be directly related to the length of the long-term variation and the location of the frequency maxima relative to the present. A test of the reversal data can be made by systematically truncating the series from the recent end, that is, if short sections of the record are removed there should be a shift in the period and phase of harmonic peaks, whereas peaks related to true periodicities in the data should not be affected.

Analyses of reversal data were made after successively removing 3-Myr portions of the record, starting at the present and moving back to 21 Myr BP, yielding a total of eight analyses. The period and phase of each peak shifts as expected for

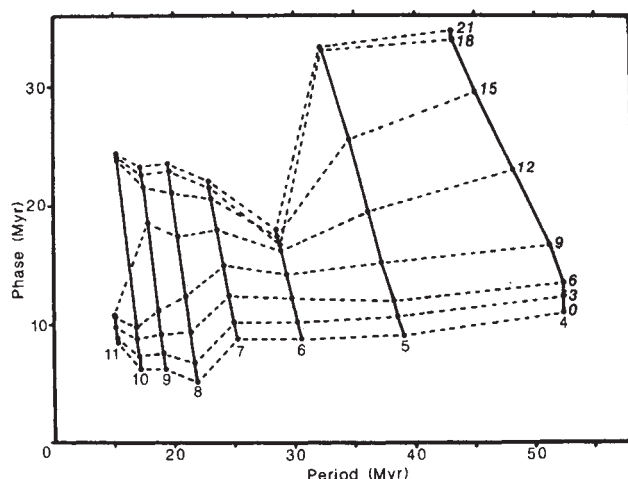


Fig. 5 Variation of phase and period of peaks that results from successive truncation of the reversal data. Dashed lines connect peaks for a single analysis; number of Myr removed is indicated at the right-hand end of each line. Solid lines show how the period and phase of each peak change. Thus, the 0-Myr line shows the period and phase of the peaks in Fig. 2*a* (the analysis of the untruncated series) and the solid lines show how each peak is affected by progressive truncation of the data set.

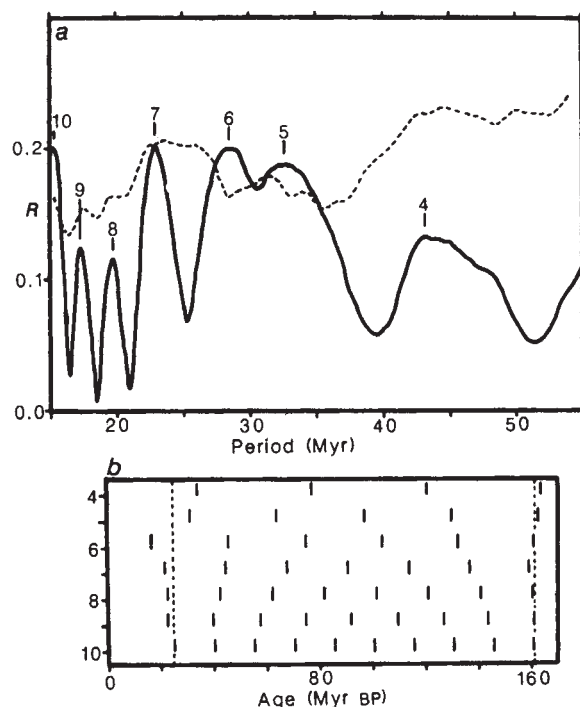


Fig. 6 *a*, Analysis of the 213 events in the reversal series truncated at 18 Myr. Dashed line, 95% confidence level based on 100 randomizations of the data. *b*, Locations of predicted events are controlled strongly by the old end of the series because the high-frequency portion of the record from 0 to 18 Myr has been almost eliminated.

harmonic peaks (Fig. 5). The period decreases systematically in response to the decreasing time span between the large frequency maxima close to the ends of the record. The phase increases systematically as a result of eliminating the most recent portion of the record. The changes in the period and phase of all of the peaks are consistent with their origin as harmonics; there is no indication of a true periodicity.

The lack of periodicity is supported by the amplitudes of peaks for the reversal record from 18 to 170 Myr ago (Fig. 6*a*). There is no longer one dominant peak but four peaks which equal or exceed the 95% confidence level (15.2, 23.0, 28.8 and

32.2 Myr). The locations of events predicted by the peaks (Fig. 6b) are controlled by the ends of the series, especially at 160 Myr as the frequency near the recent end of the record has been reduced by the truncation procedure.

Conclusions

In summary, the minimum dispersion method of analysing time series for periodicity can be performed most efficiently by means of a circular, rather than a linear, model. However, when events occur frequently throughout a predicted cycle, a correction must be made for the wrapping effect. For the magnetic reversal data this correction changes the significance levels reached by the peaks. The confidence levels determined by randomization of the observed data sequence vary as a function of period and should be calculated at each trial period.

The peaks for the reversal data originate as harmonic peaks which are controlled by the long-term variations in magnetic reversal frequency. Such control is indicated by the shift in the phase and period of all the peaks, including the 30-Myr peak, as the data are truncated. If the most recent 18 Myr of the reversal data are deleted, four peaks rather than two reach the 95% confidence level.

The dispersion between predicted and observed events is not a valid measure of periodicity when there are long-term variations in frequency on the scale of the record length. The statistical significance of a peak as determined by comparison with randomized data series is not a valid test for a periodicity in the data. Randomized distributions only provide an idea of

how 'special' the observed distribution is with regard to dispersions. Dispersion is a function of both periodicity and harmonic effects; statistically unusual dispersions (large peaks) can result from either cause. In conclusion, dispersion analysis cannot be used uncritically as a rapid means of finding periodicities in complex data sets.

Rampino and Stothers¹³ have presented dispersion analyses suggesting that periodicities of ~33 and 260 Myr dominate the Phanerozoic "geological and biological upheavals". As they suggest that variations in the frequencies of their events occur on the scale of 260 Myr it could be that the 33-Myr signal is harmonic in origin. Furthermore, the long-term periodicities are not well defined (Table 3 in ref. 13) and may simply represent long-term aperiodic variations.

Raup and Sepkoski¹⁰ apparently avoid the problems discussed here because their analysis deals only with the locations of peaks in extinction rate defined by independent means, and not with the extinction rates themselves. However, the extinction rates vary considerably with relatively high rates in the Triassic and early Tertiary but with lower average rates in the intervening Jurassic and Cretaceous. Future work which may deal directly with the extinction rates must consider the effect of this long-term variation on evidence for periodicity on the scale of 30 Myr.

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LETTERS TO NATURE

Influence of quark nuggets on primordial nucleosynthesis

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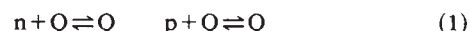
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It is possible that small droplets of quark matter, larger than ordinary nuclei, exist in nature¹. These 'nuggets' could be present in the Universe and provide one explanation for the dark matter. This suggestion is quite attractive since, unlike the case of adiabatic fluctuations of nucleons, it provides² matter fluctuations at galaxy scale and thus leads to a natural explanation for the formation of galaxies from primordial fluctuations. These nuggets, a form of baryonic matter, can be expected to interact strongly with nucleons. The question remains of whether the predicted primordial nucleosynthesis of the light elements could, in the presence of nuggets, still be consistent with the tight observational constraints. Here we examine the possibility of obtaining the observed yields of the light elements in an $\Omega = 1$ baryonic universe where a sizeable fraction of the matter is in the form of quark nuggets. Three different cases can be considered: (1) nuggets that are more stable than nuclei; (2) metastable nuggets with a long lifetime; and (3) unstable nuggets decaying into nucleons. Present models of primordial nucleosynthesis, which assume that the baryonic component of the universe is in the form of nucleons, predict abundances of D, ³He, ⁴He and ⁷Li favouring an open universe—that is, one

expanding forever. In these models the baryonic density ρ_b represents only a very small fraction of the critical density ρ_c and corresponds to a cosmological parameter $\Omega_b = \rho_b/\rho_c$ ranging from 5×10^{-3} to 0.1. If the inflation schemes³ implying $\Omega = 1$ are supported by further cosmological evidence, one might be faced with reconciling a dense universe with the results of primordial nucleosynthesis. There are, in this case, two ways to make nucleosynthesis consistent with the observations: either there is a very large fraction of non-baryonic matter (massive neutrinos or other hypothetical particles), or in a purely baryonic universe, there are non-standard nuclear reactions that modify nucleosynthesis yields. This could be the case⁴ if hypothetical massive neutrinos or gravitinos existing during the first 10^{-4} – 10^{-5} s of the universe decay into energetic photons inducing a partial photodisintegration of ⁴He into D and ³He which would be otherwise underabundant. In this study the non-standard reactions arise due to quark nuggets.

The calculation of primordial nucleosynthesis is made in the frame of the hot standard big bang. To follow the evolution of the abundances of the elements during primordial nucleosynthesis, we use a numerical code described in ref. 5. Specific reactions have been added to the standard scheme in order to describe the absorption or emission of protons and neutrons by the nuggets Q:



For baryon absorption, we use the geometric cross-section of the nugget Q plus a Coulomb barrier for protons. The emission rate of baryons by the nuggets is treated in a standard way. The fission lifetime is proportional (as it is for the fission of ordinary nuclei) to the time the emitted particle needs to cross the nugget, multiplied by the relevant energy barrier factors⁶. In the case of metastable nuggets, an energy barrier for both reactions due

to the non-zero strangeness of the quarks in the nuggets has been added. The average baryon number of a quark nugget is denoted A . This value can be obtained from the theory of quark bags⁷ but is still uncertain by many orders of magnitude. We thus take A as a free parameter of our calculation. The most probable baryon number of a nugget is A , but fluctuations may occur around this value and allow the existence of nuggets differing from the most probable one by several units of baryon number, with negligible energy cost. This in turn allows reactions (1) to occur. Secondary reactions among nuggets by changing their number will, however, ensure that their average baryon number remains the most probable one, A . In all cases of interest, equilibrium of reaction (1) is reached at $T \approx 100$ MeV, that is, after the quark-nucleon phase transition and before nucleosynthesis. Keeping the notation Ω_b for the ordinary baryonic matter, and defining Ω_{nug} as the fraction of matter in nuggets, the equilibrium abundances can be roughly evaluated from the equation:

$$\frac{d}{dt} \frac{\Omega_b}{\Omega} = \frac{\Omega_{\text{nug}}}{\Omega} \lambda_{\text{em}} - \frac{\Omega_b}{\Omega} \lambda_{\text{abs}} \quad (2)$$

The emission rate behaves as

$$\lambda_{\text{em}} \propto \eta \frac{C}{R} \frac{1}{A} \propto n_Q A^{-4/3} \quad (3)$$

where n_Q is the baryon number density ($\sim 10^{39} \text{ cm}^{-3}$) in a nugget, R the nugget radius, and $\eta \approx 0.1$. The absorption rate is estimated using the geometrical cross-section for particle absorption

$$\lambda_{\text{abs}} \sim \pi R^2 n_{\text{nug}}(v) \propto \Omega_{\text{nug}} n_c A^{1/3}$$

where n_{nug} is the number density of nuggets in the universe ($n_{\text{nug}} = \Omega_{\text{nug}} n_c / A$) and n_c ($\sim 10^{23} \text{ cm}^{-3}$) is the critical density closing the universe. For moderately large values of A , the equilibrium is thus strongly displaced in favour of ordinary baryons. For a very large average baryon number in a nugget, however, only nuggets are initially present. In practice, we start the calculation at $T \approx 100$ MeV and use all reactions (1) with the relevant rates and energy barriers, as described above. This population then quickly evolves in whatever equilibrium stage is implied by these rates.

We first consider the case of stable quark nuggets. This implies a factor $\sim e^{-\epsilon/kT}$ for the emission of a baryon where ϵ is the binding energy difference (per baryon) between quark matter and nucleons. At large enough temperatures, however, this factor is ineffective. So, for $A < 10^{15}$, only baryons are present and primordial nucleosynthesis occurs in the standard way. For $A > 10^{18}$, the equilibrium is displaced in favour of the nuggets. For $10^{15} < A < 10^{18}$, there is an intermediate situation in which both baryons and quark nuggets are present at the time of nucleosynthesis. The important role of the Coulomb barrier in this network of reactions should be emphasized. The rates for $n \rightleftharpoons p$ and $n + p \rightarrow d + \gamma$ are very large. So, as soon as nucleons are emitted by the quark nuggets, they transform into protons, and possibly into nuclei with the usual relative abundance n/p . At the lower temperatures, when the energy difference between quark nuggets and nucleons starts to play a role, quark nuggets, being the more stable element, should normally absorb the baryons again. This is prevented because all the baryonic matter is in the form of protons (or light nuclei) at this stage. The calculation of the fraction of baryonic matter emitted by the nuggets under the assumption that it cannot be transformed back into quark matter leads to:

$$\Omega_b / \Omega = 1 - e^{-x} \quad (4)$$

with:

$$x = \int_0^\infty \lambda_{\text{em}} dt = \left(\frac{A}{A_{\text{tran}}} \right)^{-4/3} \quad (5)$$

$$A_{\text{tran}} = 2.8 \times 10^{15} \left(\frac{\epsilon}{10 \text{ MeV}} \right)^{-3/2} \eta^{3/4} \quad (6)$$

that is, an exponentially rapid transition at $A \sim A_{\text{tran}}$ between

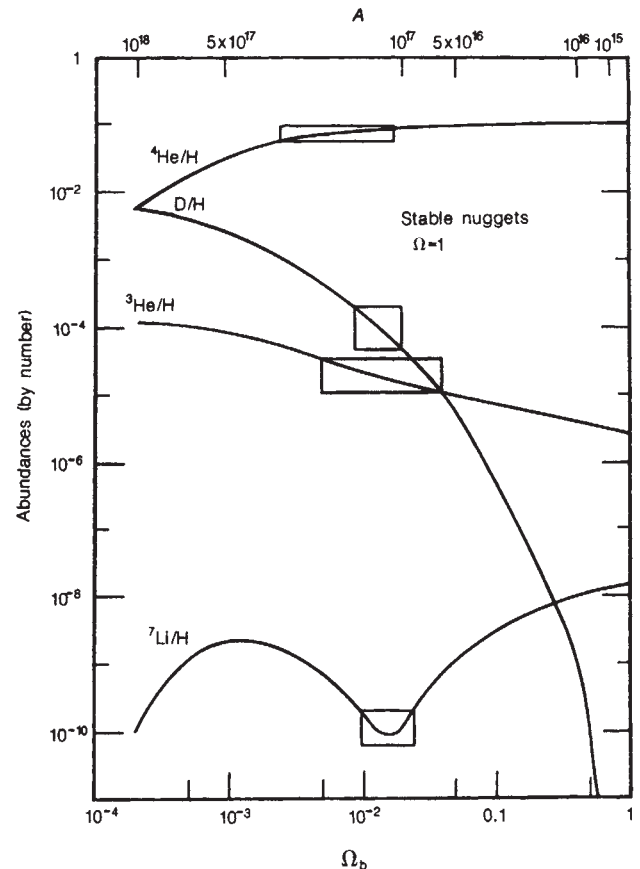


Fig. 1 Results of primordial nucleosynthesis in the case of stable nuggets with an $\Omega = 1$ universe. The fraction of baryonic matter in the form of nucleons Ω_b (lower scale) is solely determined by the size A (upper scale) of the quark nuggets—equations (4) to (6). The abundances of the light elements, which depend on the parameter A , are thus also a function of Ω_b . The boxes indicate the area allowed by observations for each of the species. All the observed abundances can be obtained simultaneously when our unique parameter A is chosen to be $\sim 10^{17}$. This leads to the prediction that $\Omega_b \sim 0.02$ and $\Omega_{\text{nug}} \sim 0.98$.

a universe made only of baryons and a universe made only of quark nuggets. This is confirmed by the actual numerical calculations. The high rate of proton and deuteron formation ensures that to a fair approximation the nucleosynthesis occurs as if one were in a universe with cosmological parameter Ω_b , the $\Omega - \Omega_b$ nuggets being just spectators. This is confirmed by a calculation shown in Fig. 1 which demonstrates the D, ^3He , ^4He and ^7Li abundances as a function of the parameter Ω_b , which is the baryon fraction of the $\Omega = 1$ universe corresponding to our choice of A . A modification of the value ϵ that determines the energy barrier is of little importance. Provided that it allows the baryons to exist at $T \approx 100$ MeV, it would merely change the relation between Ω_b and A , not that between the calculated abundances and Ω_b . We thus conclude that even for quark nuggets that are more stable than the nucleons, an appreciable fraction of matter can be in the form of baryons, and that the observational requirements $D/H \sim 4 \times 10^{-5}$, $^3\text{He}/H \sim 1.4 \times 10^{-5}$, $^4\text{He}/H \sim 0.08$, $^7\text{Li}/H \sim 10^{-10}$ can be met provided Ω_b has the proper value $\Omega_b^{(\text{NS})} = 0.02$. This implies that 98% of the matter of the universe is in the form of quark nuggets. However, it requires an adjustment of the nugget size that has to be close to A_{tran} so as to lead to the desired proportions of baryons and nuggets. This is true also for an $\Omega < 1$ universe, for which the adjustments to be made are nearly the same. Starting with $\Omega = \Omega_b^{(\text{NS})}$ is also consistent with the observed abundances, provided $A < A_{\text{tran}}$ so as to ensure that all the matter is in the form of baryons at the time of nucleosynthesis.

The case of metastable nuggets, for which there is an energy barrier slowing down the exchanges with nucleons, is rather

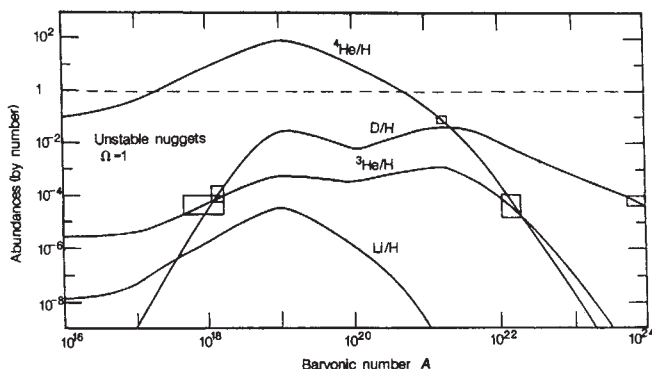


Fig. 2 Results of primordial nucleosynthesis in the case of unstable nuggets with an $\Omega = 1$ universe. The abundances of the light elements are plotted as a function of our unique parameter A , the baryonic number of a quark nugget, which characterizes the size of the latter. The boxes indicate the area allowed by observations for each of the species. There is no value of A for which all observed abundances can be obtained simultaneously.

similar to the previously discussed case, the introduction of a potential barrier having only the effect of modifying the value of ε in equation (6). If, however, these nuggets decay before galaxy formation (say, $z > 3$), the new baryonic matter that they provide should be incorporated into the yields calculated in Fig. 1. The corresponding mass fraction would then be reduced by a factor of $\sim 0.02/\Omega$, making any agreement with observations quite difficult for the ${}^4\text{He}$ abundance. On the other hand, their decay at $z < 3$ would not change the fractional abundance of the light elements in galaxies, where the observations are made. Such a high energy input (about $\varepsilon \approx 10$ MeV per baryon) at so late an epoch could have observable effects (such as temperature of the intergalactic gas).

We now consider the results (Fig. 2) for the case in which the quark nuggets are unstable and decay into nucleons. At temperatures ~ 100 MeV, an equilibrium similar to that encountered in the 'stable case' is reached between nuggets and baryons. There is, however, a temperature below which only baryons survive. For $A < 10^{16}$, this occurs before the nucleosynthesis epoch; for $\Omega = \Omega_b^{(NS)} (\approx 0.02)$, these values of A lead to the standard nucleosynthesis rates, and the standard limits on Ω still apply. For $A > 10^{22}$, on the other hand, the nuggets decay into baryons at so late an epoch that the low density of the expanding universe prevents any of the nuclear reactions from occurring. This would lead to a universe made of protons alone. For intermediate values of A such that $10^{16} < A < 10^{22}$, emission (equation (2)) by the nuggets occurs during the nucleosynthesis epoch. Neutron emission especially is important, because the Coulomb barrier for protons is rather effective and the $n \rightarrow p$ reaction rather slow. The nuclear reactions thus proceed with a n/p ratio much larger than 1, leading to ${}^4\text{He}/\text{H} > 1$. For $A > 10^{20}$, however, the baryon emission occurs late enough, at an epoch in which the density is so low that the formation of nuclei starts to be suppressed. There is obviously an epoch ($A \approx 10^{21}$) in which the ${}^4\text{He}/\text{H}$ abundance is equal to the observed value ($X_{\text{H}} \sim 0.72$, $X_{\text{He}} \sim 0.22$). Due to the lower nuclear reaction rates, however, D and ${}^3\text{He}$ are then more abundant than is usual ($X_{\text{D}} \sim 5 \times 10^{-2}$ and $X_{\text{He}} \sim 2 \times 10^{-3}$) whereas the ${}^7\text{Li}$ abundance is quite low ($X_{\text{Li}} \sim 5 \times 10^{-10}$), a value consistent with the current observations of population II stars. The overabundance of D does not constitute a major difficulty as there are scenarios of galactic evolution in which D can be thoroughly destroyed during the normal course of the history of a galaxy⁸. But a factor of ~ 100 would be difficult to obtain. The relative overabundance (two orders of magnitude) of ${}^3\text{He}$ is more problematic, since there is no obvious mechanism that would destroy this less fragile nuclear species.

Conversely, if we choose A so as to obtain an acceptable value ($\text{D}/\text{H} \approx 10^{-4}$, ${}^3\text{He}/\text{H} \approx 5 \times 10^{-5}$), the ${}^4\text{He}$ abundance is

either much too large (${}^4\text{He}/\text{H} \approx 10$ for $A = 10^{18}$) or too small (${}^4\text{He}/\text{H} \approx 10^{-4}$ for $A = 10^{22}$). A more careful examination of Fig. 2 shows that the problem is due to the existence of a fairly high plateau in the D and ${}^3\text{He}$ yield for A between 10^{18} and 10^{22} . The question must then be raised of how such a prediction depends on changes in some of our reaction rate parameters. This is still under study, but preliminary results show that this prediction is fairly insensitive to the assumptions made. This is merely due to the fact that in the presence of a source of neutrons one can only make D, then ${}^3\text{He}$ with acceptable yield when the density of the universe is low at the time the neutrons are produced. This then leads to the D and ${}^3\text{He}$ problem. The same difficulty is encountered with any scenario based on a high value of Ω . Since, however, $\Omega \sim 0.02$ leads to the standard values for the abundances of the light elements, there is obviously some region, say $\Omega \leq 1$ and nuggets with baryon number $A \approx 10^{16}$, in which the values of the calculated abundances remain acceptable.

There are, therefore, several cases in which nucleosynthesis in the presence of a primordial population of quark nuggets leads to the observed abundances of light elements. If the baryon number A , which is not really constrained by the physics of quark nuggets, is smaller than 10^{16} , nucleosynthesis occurs in the presence of nucleons only, and the standard yields as well as the known limits on Ω apply in all cases (stable, metastable or unstable nuggets). Where the baryon number of a nugget is larger, there is the possibility of having an $\Omega = 1$ universe with predicted abundances of the light elements that are comparable to the observed values in the case of stable (or possibly metastable) quark nuggets. For unstable nuggets, the calculated abundances are somewhat at variance with the observations because of their decay into nucleons during nucleosynthesis. It is important to note that these conclusions are to a large extent independent of the physics of the quark-nuclear phase transition in the early universe. Provided that equilibrium is reached at some temperatures between 10 and 100 MeV, the fraction of quark nuggets before nucleosynthesis is solely determined by the size of the nuggets and their probability of decay into nucleons. Therefore the possible existence of these quark nuggets provides another way to reconcile the early nucleosynthesis with a closed universe.

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Constraints on cosmic-ray observation of Cygnus X-3

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Two experimental groups^{1,2} working at different minimum energies have reported underground muons coming from the direction of Cygnus X-3 with rates that vary in synchrony with its binary period. The depths involved are such that the muons have energies of the order of 1 TeV for the shallower¹ and several TeV for the deeper² detector. Such observations, if verified, would require a new particle or a new physical process³. Here we explore our earlier suggestion⁴

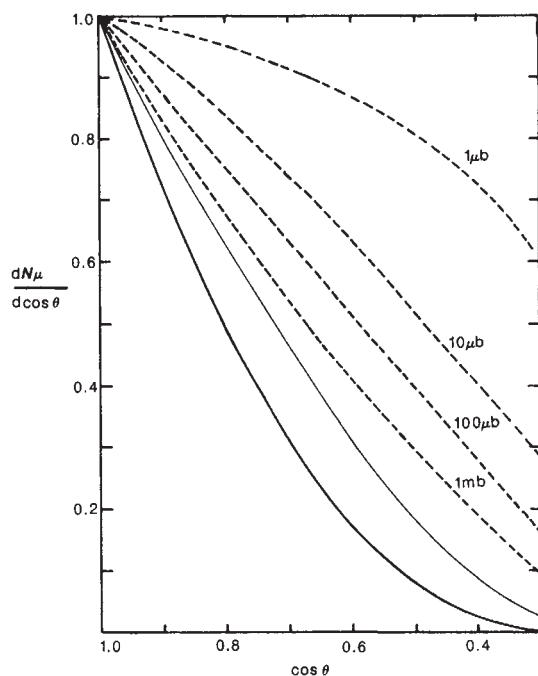


Fig. 1 Angular distribution of underground muons generated by: cosmic rays through pion and kaon decays (lower solid line); E^{-2} signal through pion and kaon decays (upper solid line); promptly with different cross-sections by E^{-2} signal (dashed lines). Depth is 2 km water equivalent (km WE); b, barn.

that the new signal carrier could be a neutral bit of quark matter, condensed and stabilized by strange quarks. We also consider other, more general explanations and conclude that it is exceedingly difficult to find a self-consistent explanation of the observations.

Cygnus X-3 is at least $L^* = 10$ kpc from Earth. This is $>10^4$ gyroradii in typical galactic magnetic fields for particles with rigidity $<10^6$ GV c^{-1} , so the progenitors of the underground muons cannot be charged particles. If these events were caused by neutrons with enough energy to reach the Earth from this distance, their flux would be easily observable in high-energy cosmic rays, where they have not been seen. Similarly, γ rays are ruled out as primaries because there are too many muons observed for them to have been generated by an acceptable number of gammas⁵ (unless gammas at high energies have unexpectedly high probabilities of producing muons). Neutrinos are eliminated as a possible primary by the substantial zenith-angle dependence of the experimental rates and by the enormous luminosity at the source that would be required to produce a signal in small detectors⁶. Therefore, if the effect is real it must be caused by some rather exotic primary.

The muons in the peak arrive within a rather narrow time period, ~ 30 min. Maintaining this time correlation gives another constraint on the particles. The time delay between the arrival of two particles which left Cyg X-3 at the same time is

$$L^*(\gamma_1^{-2} - \gamma_2^{-2})/(2c)$$

Since this time difference cannot exceed 30 min, the primaries must either be nearly monoenergetic or else the Lorentz factor must exceed 10^4 . As one increases the minimum energy of the observed particles, the last particles must be arriving earlier and the width of the peak should get smaller if the mass is near the limit. There is even some indication of such a tendency in the data^{1,2}. If the underground signal is due to muons produced in the atmosphere, the minimum energy per parent hadron must be sufficient to produce muons that can penetrate to the detector. Unless the energy is much greater than this minimum, the above constraint on the Lorentz factor then requires the mass of the parent to be less than or of the order of 1 GeV. It is difficult to believe that a long-lived particle of this mass, capable of produc-

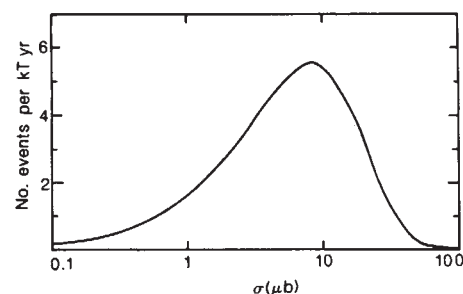


Fig. 2 Interaction rates (per ktonne yr) of signal carrier with a flux of 10^{-10} $\text{cm}^{-2} \text{s}^{-1}$ versus interaction cross-section σ . Depth is 2 km WE.

ing muons in atmospheric interactions, would have been overlooked in accelerator experiments.

One possibility is that the parent primaries might be nuggets of quark matter⁴. There are theoretical reasons for believing that such objects might exist and be stable for certain ranges of mass⁷⁻⁹. Furthermore, they might be produced in high-energy processes¹⁰ around a compact quark star¹¹. One would then expect comparable numbers of up, down and strange quarks so that stable states would tend to be nearly neutral⁹. Only charged nuggets could be accelerated, so the neutral beam would have to be produced by charge-changing collisions in the ambient matter of the source after acceleration. Correspondingly, the required power at the source must be increased by a factor $1/F$, where F is the fraction of nuggets that are neutralized by such collisions. A high content of strange quarks would lead to enhanced kaon production in the atmosphere and thus to a relatively high yield of muons. Quark globs of the right mass and energy could penetrate deep in the atmosphere and explode to give rise to Centauro events¹². A specific version of a stable ensemble of quarks has been suggested some time ago which could be relevant in the context of underground signals from Cyg X-3 (ref. 13) (but not for Centauro events). This is the di-lambda, a bound dihyperon state of $2u$, $2d$ and $2s$ quarks.

A. M. Hillas (personal communication) has pointed out that the surface air shower signal from Cyg X-3 puts a significant constraint on models which would produce the muons by interactions of nucleon-like objects in the atmosphere: assume such parent 'nucleons' are bound in aggregates of mass number A . These particles will also produce air showers. To be consistent with the observed air shower signal, dF_{surface}/dE , one then requires

$$\text{Cyg X-3 underground signal} < \int_{AE_{\mu}}^{\infty} N_{\mu}(>E_{\mu})[dF_{\text{surface}}/dE] dE$$

where N_{μ} is the number of muons per primary of total energy E that have sufficient energy to penetrate to the detector. The differential surface flux from 1 TeV (refs 14-16) to 1,000 TeV (refs 17, 18) is $\sim 4 \times 10^{-8}/E^2 \text{ cm}^{-2} \text{s}^{-1} \text{GeV}^{-1}$. Using an Elbert formula¹⁹ for underground muon yield from incident nuclei²⁰, we find a bound on the underground signal from Cyg X-3 of $1.3 \times 10^{-6} (E_{\text{GeV}})^{-2} \text{ cm}^{-2} \text{s}^{-1}$, where E_{GeV} is the minimum muon energy for the underground detector. For Soudan ($E_{\text{GeV}} = 650$), this bound is $3 \times 10^{-12} \text{ cm}^{-2} \text{s}^{-1}$ and for Mont Blanc ($E_{\text{GeV}} = 3,400$), $10^{-13} \text{ cm}^{-2} \text{s}^{-1}$. In contrast, the reported signal at Soudan is $\sim 7 \times 10^{-11}$. A flux is not stated for the signal at Mont Blanc, but an estimate can be obtained from a comparison of signal/background ratio with the background flux of single atmospheric muons in the angular region around Cyg X-3. Such an estimate gives of the order of $10^{-11} \text{ cm}^{-2} \text{s}^{-1}$. Thus the underground signal appears to be at least a factor 20 too high to be induced by nucleons. Conversely, the parent hadrons must be at least 10-20 times more prolific at producing muons relative to air showers than are nucleons. In view of the quark matter suggestion (for which kaon and hence atmospheric muon production should be enhanced), we ran the cascade simulation of ref. 20 for incident lambda hyperons, forcing production of a leading kaon at each lambda interaction. The muon production was enhanced by a factor <2 relative to nucleons, so even in

this case there is a problem of consistency with the surface air shower fluxes.

A conceivable way out is to arrange the interaction length of the parent to be comparable with or greater than the thickness of the atmosphere so that production of the signal occurs too low for air shower production (that is, mostly in the Earth). In this case, however, muon production must be prompt, which would require a signal carrier other than a quark nugget.

One can, in principle, use the energy-dependence of the signal implied by the different depths of the experiments to determine whether the muon production is prompt or atmospheric through pion and kaon decay. In the latter case, the signal should be suppressed by an extra power of E_{GeV} as the depth increases due to time dilation of the parent pions and kaons. If the spectrum of the carrier from the source is E^{-2} (differential) one would expect the ratio Soudan/NUSEX underground signal = 5 for prompt, and $\approx (5)^2$ for atmospheric pion and kaon decay. The ratio of the observed fluxes quoted above is closer to 5, but the analysis is inconclusive because we have not taken account of the complex variation of the overburden in the line-of-sight to Cyg X-3 as it passes across the sky at Mont Blanc.

Assuming that the underground muons are produced by interactions of a new particle in the rock, one can ask what the cross-section would have to be to avoid producing too many contained events in the nucleon decay detectors (J. C. van der Velde, personal communication). A separate constraint is the observation that the angular dependence of the signal is similar to that for atmospheric muons. Thus, production must be near the surface, which places a lower limit on the cross-section. Figure 1 shows the angular distribution of underground muons produced in the atmosphere through pion and kaon decay compared with the distributions expected for prompt production with various cross-sections. The smaller the cross-section, the more nearly isotropic is the angular distribution of the signal because the muon production occurs deeper in the rock.

Similarly, the rate of contained interactions that originate in a large underground detector can be plotted as a function of cross-section. For large cross-sections the deep detector is shielded by the rock. For very small cross-sections the interaction rate per unit volume becomes too small for detection. The resulting interaction rate is shown in Fig. 2. From Figs 1, 2 it appears that consistency with the observations requires a cross-section for prompt muon production $> 0.01 \text{ mb}$ (10^{-30} cm^2).

Even if a new neutral particle with appropriate properties is postulated, one is still left with the problem of explaining the fact that the signal in ref. 2 has an angular width several times the resolution of the detector. If one attempts to explain this by production of secondary muons with characteristically large transverse momentum, one would expect the events at the shallower detector to be even more spread out, whereas the opposite is observed (L. D. McLerran, personal communication). It appears difficult, therefore, to account for the observation of signals from Cyg X-3 deep underground.

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Photinos from cosmic sources

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In supersymmetric (SUSY) theories¹, the photino is the fermion partner of the photon and is expected to be the lightest stable particle. Despite many discussions of the possible production of photinos, and other supersymmetric particles, at existing and proposed accelerators², so far, experiments have only set limits^{3,4}. We propose here that photinos are produced by sources of ultra-high-energy cosmic rays. We specifically consider Cygnus X-3, which is a powerful source of neutral particles up to 10^4 TeV (refs 5–8). These are usually assumed to be γ rays, but there is no direct evidence for this, and shower^{5,6} and underground^{9,10} experiments suggest that there may be another component present. Here we estimate that a photino flux of the order of $10^{-14} \text{ cm}^{-2} \text{ s}^{-1}$ above 10 TeV is possible from a source such as Cyg X-3, under certain conditions, and consider the feasibility of detecting these photinos.

Whatever the neutral particles, they probably result from the decay of hadrons produced by protons accelerated by a compact object, such as a neutron star, and striking a considerable thickness of matter ($\sim 50 \text{ g cm}^{-2}$) along the line-of-sight to Earth¹¹. Photinos would occur by the production and decay of gluinos. Several factors make this proposal feasible. First, the cross-section for the production of pairs of gluinos in proton-proton (pp) interactions is relatively strong, depending on the gluino mass, and rises rapidly with energy^{12–14}. The gluino decays into a photino plus a quark pair before interacting. The photino can readily escape the matter surrounding the source, but may have a sufficiently high cross-section for detection at the Earth to be feasible.

In a previous paper¹¹, the one-dimensional diffusion equation was used to calculate the γ rays and neutrinos which would emerge from a source of very high-energy protons, through the production and subsequent decay of pions in the matter along the line-of-sight to Earth. Here we follow a similar procedure, applying the same equations to gluinos and photinos by analogy.

The source is assumed to emit protons with a differential power law spectrum $N_p(E_p) = N_p(1)E_p^{-\alpha}$, with E_p the proton energy in TeV, cutting off at E_p^{max} , where $N_p(E_p)$ is the rate at which protons of energy E_p are emitted, per unit E_p . These protons produce gluinos of energy $E_{\tilde{g}}$ by gluon fusion in the reaction $pp \rightarrow \tilde{g}\tilde{g}X$ (refs 12–14), where X are other hadrons. Because they are likely to have a sufficiently short lifetime to decay before interacting, the gluinos which emerge from a matter depth $z \text{ g cm}^{-2}$ will be given by a formula analogous to that for pions (π^0) in ref. 11 such that

$$N_{\tilde{g}}(E_{\tilde{g}}, z) = n_A N_p(1) E_{\tilde{g}}^{-\alpha} \Lambda_{\tilde{g}} e^{-n_A \sigma_{\tau} z} g_{\tilde{g}}(E_{\tilde{g}}) \quad (1)$$

where $\Lambda_{\tilde{g}}$ is the gluino decay length, σ_{τ} is the total pp cross-section (assumed constant), n_A is Avogadro's number and

$$g_{\tilde{g}}(E_{\tilde{g}}) = \int_{x_{\text{min}}}^1 x^{\alpha-1} \frac{d\sigma_{\tilde{g}}}{dx} \left(\frac{E_{\tilde{g}}}{x}, x \right) dx \quad (2)$$

where $x = E_{\tilde{g}}/E_p$, with $x_{\text{min}} = E_{\tilde{g}}/E_p^{\text{max}}$, and the integration over x is equivalent to the integration over E_p . In ref. 11, we use Feynman scaling to compute g_{γ} and g_{ν} , the corresponding quantities for γ rays and neutrinos, independent of energy. Here the gluinos are produced centrally and close enough to threshold for this approximation to be invalid, so $g_{\tilde{g}}$ will still be a function of $E_{\tilde{g}}$. Let us assume that the gluinos are produced on the rapidity plateau, so $d\sigma_{\tilde{g}}/dx \propto x^{-1}$. Let us also assume that the differential spectral index $\alpha = 2$, as suggested by observations and models for cosmic ray acceleration. Then it is easy to show that

$$g_{\tilde{g}}(E_{\tilde{g}}) = \sigma_{\tilde{g}}(E_{\tilde{g}}) \frac{[1 - E_{\tilde{g}}/E_p^{\text{max}}]}{\ln(E_p^{\text{max}}/E_{\tilde{g}})} \quad (3)$$

where $\sigma_{\tilde{g}}(E_{\tilde{g}})$ is the total gluino production cross-section at a proton energy $E_{\tilde{g}}$.

The photinos which emerge from the matter around the source are governed by a diffusion equation analogous to that for γ rays in ref. 11, except that re-interaction can be neglected. The rate of photinos at an energy $E_{\tilde{\gamma}}$ then becomes, in the thick source limit $n_A \sigma_T z \gg 1$,

$$N_{\tilde{\gamma}}(E_{\tilde{\gamma}}) = N_p(E_{\tilde{\gamma}}) \frac{1}{\sigma_T} \int_{y_{\min}}^1 y^{\alpha-1} g_{\tilde{g}} \left(\frac{E_{\tilde{\gamma}}}{y} \right) dy \quad (4)$$

where $y = E_{\tilde{\gamma}}/E_{\tilde{g}}$, with $y_{\min} = E_{\tilde{\gamma}}/E_p^{\max}$. Isotopic decay in the centre-of-mass has been assumed.

Thus, we can write $N_{\tilde{\gamma}}(E_{\tilde{\gamma}}) = \varepsilon_{\tilde{\gamma}}(E_{\tilde{\gamma}}) N_p(E_{\tilde{\gamma}})$, where

$$\varepsilon_{\tilde{\gamma}}(E_{\tilde{\gamma}}) = \frac{1}{\sigma_T} \int_{y_{\min}}^1 \sigma_{\tilde{g}} \left(\frac{E_{\tilde{\gamma}}}{y} \right) \frac{[y - y_{\min}]}{\ln(y/y_{\min})} dy \quad (5)$$

is the 'efficiency' for protons with an E_p^{-2} spectrum to produce photinos of energy $E_{\tilde{\gamma}}$.

We use the atmospheric Cerenkov observations around 1 TeV, which are probably γ rays, to estimate $N_p(E)$. The differential γ -ray flux at energy E observed from the source at a distance R will be

$$F_{\gamma}(E) = \varepsilon_{\gamma} D_{\gamma} N_p(E) \frac{\Delta\Omega}{4\pi R^2} \quad (6)$$

where D_{γ} is the duty factor for the γ -ray pulse, $\Delta\Omega$ is the solid angle of the proton beam, and ε_{γ} is the 'efficiency' for protons with a power law spectrum to produce TeV γ rays. In the most optimum conditions for γ -ray production, that is, a column density of $\sim 50 \text{ g cm}^{-2}$, ε_{γ} is estimated in ref. 11 to be a constant 2.5% for an E_p^{-2} proton spectrum, with the resulting γ -ray spectrum having the same power law. Other calculations give as much as 10% (ref. 15) but lower efficiencies, and thus higher estimates of $N_p(E)$, will result when the column density is not optimum for γ -ray production or when other photon absorption processes are present. (In principle, all γ -ray attenuation along the line of sight is included in ε_{γ} .) We can then estimate the photino flux at an energy E from the same source to be:

$$F_{\tilde{\gamma}}(E) = F_{\gamma}(E) \frac{D_{\tilde{\gamma}} \varepsilon_{\tilde{\gamma}}(E)}{D_{\gamma} \varepsilon_{\gamma}} \quad (7)$$

where the beam solid angle and distance cancel, but the duty factor for photinos may be different from that for γ rays.

The observed γ -ray flux from Cyg X-3 above 1 TeV is $F_{\gamma}(E) = 3 \times 10^{-11} E^{-2} \text{ cm}^{-2} \text{ s}^{-1} \text{ TeV}^{-1}$, cutting off above 10^5 TeV (ref. 16). Using this in equation (7), with $\varepsilon_{\gamma} = 2.5\%$, $E_p^{\max} = 10^5 \text{ TeV}$, and assuming equal duty factors, the resulting photino flux is shown in Fig. 1, where it is compared with the γ -ray flux. We see that the photino flux is about 10% of the γ -ray flux above 100 TeV when the gluino mass $M = 2\text{--}3 \text{ GeV}$, near the current experimental limit^{3,4}. Higher gluino masses will yield correspondingly lower fluxes, so this is clearly one critical parameter. The total flux integrated over energy is $3 \times 10^{-14} \text{ cm}^{-2} \text{ s}^{-1}$ for $M = 2 \text{ GeV}$, about three orders of magnitude lower than the total γ -ray flux above 1 TeV. So the main photino effect can be expected only at 100–1,000 TeV. Because the observed Cyg X-3 neutral particle flux cuts off above 10^5 TeV , fluxes at these levels are not ruled out by recent limits on deeply penetrating particles from the Fly's Eye experiment¹⁷.

There are circumstances which could raise the flux level well above these estimates. For example, if there is significant γ -ray attenuation the source luminosity will be higher than that which is estimated from the γ -ray flux; astrophysical considerations do not rule out proton luminosities 1,000 times higher. Also, models of the Cyg X-3 powerhouse (refs 18–23 and V. Berezhinski, G. Castagnoli and P. Galeotti, unpublished calculations) suggest that the duty factor of the neutrino pulse will be perhaps 20 times higher than that for γ rays; the same arguments would apply for photinos.

The detectability of photinos at the level of Fig. 1 may be feasible because of the semi-strong nature of the photino interaction with matter¹². The cross-section, while small, can be greater

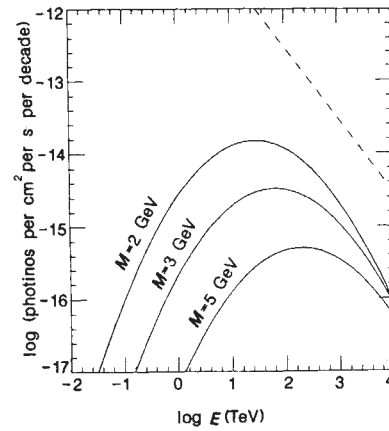


Fig. 1 The predicted flux spectrum of photinos from Cyg X-3 using the proton luminosity obtained from γ -ray observations, with the conservative assumption that conditions are optimal for γ -ray production and that the γ rays are not attenuated, for three gluino masses M . The dashed line is the observed flux of γ rays.

than that for neutrinos, which are expected to be produced at comparable levels¹¹. The photino interacts with a quark in the nucleus through squark exchange, producing a gluino which quickly decays into a photino and a quark pair. The cross-section depends strongly on the squark mass μ , falling off as μ^{-4} ; at 100 TeV it is $\sim 1 \text{ microbarn}$ (10^{-34} m^2) for $\mu = 10 \text{ GeV}$, when the squark propagator is properly inserted in the published^{12–14} formula. Because the atmosphere is $1,000 \text{ g cm}^{-2}$ thick there is a very small probability that the photino initiates an air shower before reaching the ground. However, such events would be distinctive, occurring low in the atmosphere and producing more muons than a typical electromagnetic shower. Using the photino flux from Fig. 1, the integrated flux of air showers at the surface of the Earth has been calculated to be $1 \times 10^{-17} \text{ cm}^{-2} \text{ s}^{-1}$ over all energies and $1 \times 10^{-19} \text{ cm}^{-2} \text{ s}^{-1}$ above 1,000 TeV. These are well below the reported air shower flux from Cyg X-3 of $3 \times 10^{-14} \text{ cm}^{-2} \text{ s}^{-1}$ above 1,000 TeV (refs 5, 6). It would seem that a photino interpretation for the observed showers, which do appear anomalous in at least one experiment^{7,8}, is unlikely.

For higher squark masses the photino cross-section is lower and atmospheric interactions become even more unlikely. However, even in that case there may be a reasonable chance for interaction in the Earth. Unfortunately we cannot expect too many secondary muons; any produced pions and kaons are more likely to interact before decaying, so we must rely on prompt production, that is, the production and semi-leptonic decay of heavy quarks. Prompt muon production in pp interactions occurs at about the 10^{-4} level at accelerator energies and is possibly as high as 10^{-3} in higher energy cosmic rays²⁴. There is some indication at the CERN collider that charm production is much higher than predicted from standard model calculations²⁵, and there are some arguments for greater heavy quark production in SUSY interactions which need further study. So perhaps prompt muon production by photinos at 1,000 TeV will be enhanced.

Figure 2 shows the estimated underground muon flux from photinos, as a function of slant depth. The photino flux for $M = 2 \text{ GeV}$ in Fig. 1 was assumed at the surface, multiplied by a factor $(\tilde{\gamma}/\gamma) (\mu/\tilde{\gamma}) = 0.1$. This could result, for example, if the proton luminosity is a factor of 30 higher than estimated from γ rays and prompt muon production is a factor of 30 higher than in pp collisions at accelerator energies, not impossible with our current state of knowledge. The results can be scaled by this factor, or reduced for higher gluino masses in the proportion of Fig. 1.

As seen in Fig. 2, the muon flux builds up with depth, peaking at about 5,000 m water equivalent. A distinctive signature occurs for the lower gluino mass: the flux falls off below the horizon. The flux level, with the assumptions made here, is too low to explain the underground muon events reported from Cyg X-3

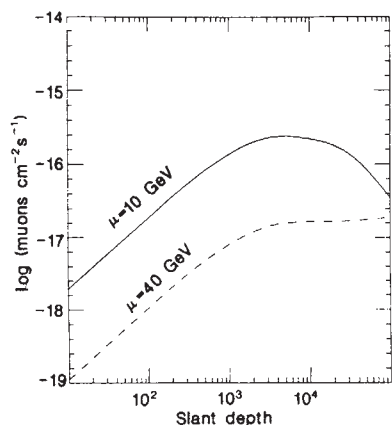


Fig. 2 The flux of photino-initiated muons underground when the photino flux is as shown in Fig. 1, for gluino mass $M = 2$ GeV, two squark masses, and $(\tilde{\gamma}/\gamma)(\mu/\tilde{\gamma}) = 0.1$, as a function of slant depth (given in m water equivalent).

(refs 9, 10), but would result in 12 events per year in the proposed undersea detector, DUMAND.

Sources other than Cyg X-3 may be more promising. For example, Hercules X-1 has now been confirmed as a strong source of γ rays from 1 TeV (refs 26, 27) to 10^4 TeV (ref. 28) LMCX-4 has also been observed at the highest energies²⁹, and is far enough away for there to be substantial γ -ray attenuation.

Thus, it is not inconceivable that photinos, if they exist, will be observed from astronomical bodies. However, this discussion shows that there are certain requirements if this is to happen in the near future. First, the source must have a proton luminosity close to its astrophysical limit. Second, there will have to be conditions between us and the source which reduce the flux of γ rays; otherwise, much higher fluxes would exist than is evident. Third, the gluino and squark masses must be close to the current experimental limits.

Let us consider the last point further. The lower limit on squark masses is ~ 12 GeV for charge $1/3$ and 15 GeV for charge $2/3$ from e^+e^- collisions^{3,4}. Beam dump experiments place a lower limit on the gluino mass of 4–5 GeV for a squark mass of 10–20 GeV, with lower gluino masses possible for higher squark masses^{30,31}.

The observation of monojets with large missing transverse momentum p_T at the CERN $\bar{p}p$ collider³² has received considerable attention as possible evidence for SUSY. If the SUSY interactions except at certain energies where a squark is resurposes) two possible scenarios for gluino and squark masses^{33–35}. In the first, each has a mass of ~ 40 GeV and measurable photino rates from cosmic sources can probably be ruled out. In the other, the gluino mass is ~ 3 GeV and the squark mass ~ 100 GeV. This latter case could lead to a significant photino flux from Cyg X-3, but the photino cross-section would probably be too low for atmospheric or underground interactions except at certain energies where a squark is resonantly produced.

However, there is still debate over whether the monojets cannot be given a non-SUSY explanation. If they can, the conventional wisdom is that the gluino and squark masses, for the scenarios discussed above, then become lower limits. But the published data³² suggest that there may still be an experimental window at low gluino and squark masses where the p_T carried by the photino is not sufficient for the event to pass the experimental cuts. In that case, gluino production would be large, but the SUSY events would be indistinguishably buried in the huge mass of ordinary data. While this window is small, and there are some theoretical arguments against its existence³⁶, it does not seem that it can yet be ruled out experimentally.

In conclusion, measurable levels of photinos from cosmic sources such as Cyg X-3 cannot yet be ruled out by current

theoretical or experimental knowledge. If the anomalous air showers and underground muons reported from Cyg X-3 are confirmed by further observation, then no conventional explanation, including neutrinos, will suffice. A photino explanation would at least not be *ad hoc*.

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The statistical significance of close pairs of QSOs

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Burbidge, Burbidge and O'Dell¹ have proposed a method of statistical analysis of close pairs of quasistellar objects (QSOs) to test the null hypothesis that the redshifts of QSOs are of cosmological origin. Only two close pairs of QSOs were then known, therefore the analysis was inconclusive. Since then a number of close pairs of QSOs have been discovered and the same statistical test can be rediscussed with a greater measure of confidence. Indeed, as we show here, the probability of finding so many close pairs by chance, as required by the cosmological hypothesis, is as small as $\leq 10^{-4}$, depending on observational uncertainties.

Close pairs of QSOs have conventionally been placed in three categories, each implying a very different physical interpretation. (1) Pairs with very small angular separation (≤ 7 arc s) but with identical redshifts are interpreted as gravitationally lensed images of a single object; (2) pairs or triplets with similar but not identical redshifts are considered members of a supercluster; (3) pairs with very different redshifts. These are either ignored or considered to arise as a chance projection effect of no physical significance.

Thus, the way in which we treat a particular close pair of QSOs seem to be dictated by the values of redshifts of its two

Table 1 Close pairs of QSOs with separation <120 arc s and with at least one member a radio source

Sources	Radio catalogue nos	Flux densities (in Jy)	Redshifts	Magnitudes	Separation (arc s)	Refs
0240+011 (BSO 2)	—	—	0.599	18.8	BSO 2-RSO = 96	7,8
0240+011 (BSO 1)	—	—	1.945	19.8	BSO 1-RSO = 115	
0241+011 (RSO)	PKS	$S_{2,700} = 0.122$	1.411	20		
0254-334 A	PKS	$S_{2,700} = 0.37, S_{5,000} = 0.53, 0.42$	1.915	17.00	54	6, 9, 10
0254-334 B	—	—	1.857	16.00		
1038+528	OL 564	$S_{966} = 0.8, S_{1,415} = 0.44$	0.677	17.5	33	6, 11, 12, 13
1038+528	—	—	2.30	18.5		
1211+334 A	ON 319	$S_{408} = 1.65, S_{1,415} = 1.13$	1.598	17.00	81	6, 14, 15
1211+334 B	—	—	1.818	20.5		
1349-439 A	PKS	$S_{2,700} = 0.54, S_{5,000} = 0.76$	?	18.5	54	6, 16
1349-439 B	—	—	0.323	17.50		
1532+016	PKS	$S_{2,700} = 1.08, S_{5,000} = 0.94$	1.42	18.0	52	17, 18, 19
1532+016	—	—	0.310	17.0		
1545+210	3C 323.1	$S_{2,700} = 1.29, S_{5,000} = 0.92$	0.264	16.69	113	20, 21, 22, 23
1545+210	—	—	(1.89)	19.5		
1548+114 A	4C 11.50	$S_{178} = 3.9$	0.436	17.00	5	6, 24, 25
1548+114 B	MC 2	$S_{408} = 2.5$	1.901	19.00		
1721+343	4C 34.47	$S_{178} = 5$	0.206	16.50	60	6, 26
1721+343	—	—	1.80	19.0		
2143-156 A	PKS	$S_{2,700} = 1.11, S_{5,000} = 0.82$	0.700	17.50	78	6, 27
2143-156 B	—	—	2.055	18.50		
2217+087	4C 08.66	$S_{1,413} = 0.16, S_{4,873} = 0.070$ $S_{178} = 2.1$	0.6227	18.00	99	28, 25
2217+087	4C 08.66	$S_{1,413} = 0.20, S_{4,873} = 0.075$	0.2282	18.00		
2320-035	PKS	$S_{2,700} = 0.42, S_{5,000} = 0.39$	1.410	18.60	78	6, 17, 10
2320-035	—	—	2.040	20.60		

members. For example, according to the conventional view, the close pair 1548+114 A,B, with angular separation 5 arc s, is placed in category (3) because its member redshifts 0.436 and 1.901 are very different, whereas the close pair 0957+561 A,B, with angular separation 5.7 arc s and identical redshifts 1.407, 1.407, is placed in category (1).

This procedure can be defended if the density of the QSO population is such that QSOs with different redshifts (and hence different distances, according to the cosmological hypothesis) are frequently projected close to each other for any randomly chosen observer in the Universe. When the first two pairs were found—Ton 155 and 156, and 1548+114 A,B, with discrepant redshifts and small separations of 35 and 5 arc s, respectively—statistical arguments were given^{2,3} to show that their occurrence by chance was very unlikely. However, these results were discounted on the grounds that *a posteriori* computation of probabilities can be misleading.

To avoid such a criticism, Burbidge, Burbidge and O'Dell¹ made *a priori* predictions of the expected number of close pairs arising by accident in any future survey of QSOs. Their expected number of QSOs lying within θ arc s of an arbitrary search centre is given by

$$\langle n \rangle = 2.4 \times 10^{-7} \Gamma(<m) \theta^2 \quad (1)$$

where $\Gamma(<m)$ is the sky density of QSOs brighter than magnitude m , expressed in units of (arc deg)⁻². Thus, $\langle n \rangle = 1$ when $\Gamma = 1$ and $\theta = 3,600/\sqrt{\pi}$. This formula can be applied at any time to decide whether the observed number of close pairs of QSOs is consistent with that expected by chance projection effects.

A search around each centre may be considered a 'trial' and the discovery of a pair a success. If there are N trials, then the expected number of successes is given by

$$\langle S \rangle = N \langle n \rangle = 2.4 \times 10^{-7} \Gamma N \theta^2 \quad (2)$$

If the cosmological hypothesis is correct, then the probability of observing at least r pairs in N such trials can be estimated by using the Poisson distribution. The hypothesis becomes of doubtful validity if the observed number r is such as to make the probability very small, say $<10^{-2}$.

To avoid the criticism of being *a posteriori*, the choice of θ in formula (1) must be *ad hoc*, in the sense that it should not

bear any relation to the close pairs already discovered. In the earlier studies (for example, refs 2, 3), the value of θ was equated to the separation of the close pair under investigation, and it was argued that the probability of an event cannot be unambiguously determined after it has occurred, in terms of its observed parameters.

We investigate here the close pairs in which at least one member QSO is a radio source. Table 1 lists all such close pairs of QSOs, excluding those that are accepted as candidates for gravitationally lensed objects. As radio positions used in optical identification usually come with error boxes of sides ≤ 10 –20 arc s, the value of θ chosen in formula (1) should be large compared with these values. On the other hand, θ cannot be too large if our whole purpose is to test whether the apparent closeness is significant enough to warrant physical association between the members of the pair. These considerations suggest that θ could be chosen in the range ~ 60 –180 arc s. Here we use $\theta = 120$.

We have found a total of 30 pairs with $\theta \leq 120$ in the literature, of which 6 have almost identical redshifts and are thought to be gravitational lenses. In all the other cases the two redshifts are different.

Because observationally, it is easier to pick out bright pairs, the test is more meaningfully applied for the brighter QSOs. Thus, we arbitrarily set our faintness limit at $V = 18.5$, as this value is bright enough for detection and faint enough to ensure enough 'trials' to make the application of formula (1) statistically viable. Although QSO catalogues list the V magnitudes, the surface densities $\Gamma(m)$ are generally given for the B magnitudes. Taking average $\langle B - V \rangle = 0.3$, we therefore set $m = 18.8$. Studies of surface densities⁴ suggest that $\Gamma \leq 2$ at $m = 18.8$. With $\theta = 120$, $\Gamma = 2$, formula (2) gives

$$\langle S \rangle = N \langle n \rangle = 7 \times 10^{-3} N \quad (3)$$

What is the value of N ? Using the criterion specified above, N is the number of radio loud QSOs brighter than $V = 18.5$ whose neighbourhoods up to 120 arc s have been carefully scanned for close companion QSOs brighter than $V = 18.5$. The total number of QSOs listed in the catalogue of Véron-Cetty and Véron⁵ which are radio loud and brighter than $V = 18.5$ is $\sim 1,030$. The corresponding number in the Hewitt-Burbidge catalogue⁶ is ~ 850 .

We will set $N = 10^3 f$, where f indicates the fraction of such QSOs whose fields have been searched for close companions out to $\theta = 120$.

In practice no such systematic exercise has been undertaken. The optical identification programme does involve a search for an optical candidate near the radio position, but usually such a search is stopped when a source is found within the error box of the radio position. Taking this error box as $\sim (20 \text{ arc s})^2$, we note that this is only $\sim 10^{-2}$ of the total area $\pi\theta^2 \text{ (arc s)}^2$. This indicates the value for f , although we will take it as high as 0.1 (meaning that about 100 radio QSOs have been carefully searched for close pairs out to 120 arc s). We then obtain from formula (2)

$$\langle S \rangle = 0.7 \quad (4)$$

It is clear from Table 1 that there are six close pairs that meet our pre-set criteria. The Poisson probability of obtaining 6 'successes' with a mean of 0.7 is $\leq 1.3 \times 10^{-4}$. This probability is too low to support the belief that these pairs arise from the chance projection of cosmologically distant objects in neighbouring directions.

In principle, formula (1) can be applied to optically selected QSOs also. There are four pairs of radio-quiet QSOs of very different redshift with $V \leq 18.5$ and $\theta < 120$. However, N cannot be estimated very reliably for radio-quiet objects.

Although our analysis indicates that very close pairs of QSOs with different redshifts are physically associated in space, we advocate considerable caution in making this a firm conclusion because of its profound implications for cosmology. The test itself is *a priori* in laying down the prescription in terms of observable parameters like Γ , m , f and θ . However, critics could argue that our chosen estimates of Γ and f have observational uncertainties. For example, if f were as high as 1, $\langle S \rangle$ in formula (4) is raised to 7 and the significance of the result disappears. We do not question this point of view and are happy to record it here because it demonstrates the vulnerability of our approach to observational checks. Whether our estimates and our conclusion are correct can easily be judged when we have better estimates of Γ and m and after the θ neighbourhoods of QSOs have been systematically searched.

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Shock-induced star formation in G357.7–0.1?

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Recent studies using the Very Large Array (VLA) telescope have revealed the existence of two unusual extended non-thermal sources, G357.7–0.1 and G53–1.0 (refs 1, 2). Classified earlier as supernova remnants (SNRs), these sources have a marked axial symmetry and compact sources on the axis at one edge. We have now looked for counterparts of these sources in the Infrared Astronomical Satellite (IRAS) Point Source Catalogue and find an IRAS source showing the characteristic spectrum of an early type star embedded in a dusty cloud, coincident with the compact source in G357.7–0.1. We propose that the compact object is an O star whose formation was triggered by the shock front of the diffuse radio source.

The two radio sources have recently been studied at wavelengths of 20 cm and 6 cm using the VLA^{1,2}. Both have unusual axial symmetry, non-thermal spectra, significant linear polarization and compact sources at one edge on the axis. The compact source near G357.7–0.1 has a flat spectrum with flux densities of $30 \pm 10 \text{ mJy}$ (ref. 1) ($60 \pm 5 \text{ mJy}$; ref. 2) at 20 cm and $48 \pm 14 \text{ mJy}$ (ref. 1) ($44 \pm 5 \text{ mJy}$; ref. 2) at 6 cm. The size of the source is $\sim 6 \text{ arc s}$ (ref. 3). To understand the nature of the compact sources and their relationship to the diffuse sources, we looked for their counterparts in the IRAS Point Source Catalogue. Although we did not find a source associated with the compact source in G53–1.0, we found an IRAS source (17368–3057) within 5 arc s of the position of the compact source in G357.7–0.1. This has also been independently reported by Shaver *et al.*³. The coordinates of the IRAS source are RA (1950) = 17 h 36 min 52.1 s and dec. (1950) = $-30^\circ 57' 17''$, with the positional error ellipse having semi-major and semi-minor axes of 21 and 10 arc s, respectively, and a position angle of 106° . The flux densities (Jy) in the four IRAS bands of 12, 25, 60 and 100 (μm) are 1.67, 6.1, 118 and 232, respectively. The uncertainties in the flux densities in the four bands are 14, 15, 16 and 18%, respectively. The source, being close to the galactic centre, has been processed through the IRAS high source density processor⁴. The point source correlation coefficients for the 12-, 25-, 60- and 100- μm bands are 98, 100, 97 and 100%, respectively. There is no 'weeks-confirmed' small extended source in the neighbourhood. However, the number of unconfirmed (only seconds-confirmed) sources are 1, 4, 5 and 3 in the 12-, 25-, 60- and 100- μm bands, respectively, indicating that the source may not be completely point-like and isolated.

The probability of chance coincidence of the radio and IRAS source is quite small ($\sim 2 \times 10^{-5}$ from radio source count data of galaxies⁵). The extended source is unlikely to be extragalactic^{1,2}. Assuming, then, that it is a galactic object, is the compact source associated with it? To estimate the frequency of occurrence of sources with infrared spectra similar to that of G357.7–0.1, we counted sources satisfying the following criteria: (1) the source must have definite flux density measurements for the 60- μm band and for at least one other band; (2) the 60- μm flux density be $> 100 \text{ Jy}$; and (3) the flux density at 60 μm must be greater than that at 25 μm . We counted sources in a rectangular strip centred on G357.7–0.1 and having length and width of 5° galactic longitude and 2° galactic latitude, respectively, and found the source density to be 3.7 deg^{-2} . The compact source is seen at the more intense western edge (6 arc min long) of the diffuse source. The probability that the compact source is found along this edge within a strip of width 40 arc s (the same as the major axis of the IRAS positional error ellipse) is then 4×10^{-3} . The fact that the source is along the axis of symmetry further reduces the probability. Thus, it seems unlikely that the compact source is not associated with the diffuse source.

The infrared spectrum of the compact source is typical of an

early OB star embedded in a dusty cloud in which the far-infrared-emitting dust is heated by the radiation from the central star as well as by the near and mid-infrared radiation from the inner dust shells⁶. Adopting a distance of 10 kpc for the source^{1,2} and applying colour correction to the catalogued values, the integrated luminosity in the 8–116- μm band is $2.3 \times 10^4 L_{\odot}$. The flux value of 100 μm is uncertain because of the contribution from cirrus clouds. However, the 100- μm band contributes only a third of the integrated luminosity. The dust temperature implied by 25-, 60- and 100- μm band flux densities varies between 40 and 60 K depending on whether the emission is optically thick, or thin with an emissivity dependence of λ^{-1} . For this temperature range, the fraction of the total luminosity outside the 8–116 μm band is ~ 0.5 . Thus, the total luminosity is $4.6 \times 10^4 L_{\odot}$, corresponding to that of a ZAMS O9 star⁷. For such a star, the ratio of bolometric luminosity to the luminosity in the Lyman continuum photons, which excite the HII region, is⁷ ~ 10 . The observed radio spectrum is consistent with nearly optically thin free-free emission at 6 cm. Using the 6-cm flux density, we then estimate the ratio of total infrared flux to the Lyman continuum flux to be 25. This will be consistent with the expectation for an O9 star assuming that about 40% of the Lyman continuum photons are absorbed by the gas⁸. Thus, the observed spectrum and the ratio of infrared to radio flux argue strongly for the presence of an early star.

We propose that the formation of the O star has been triggered by the advancing shock front of the diffuse object. If the extended object is a SNR, its age is in the range of 1,000–2,000 yr (ref. 9), too short compared with the $\sim 25,000$ yr needed for the contraction of an O9 star (mass of $25 M_{\odot}$) and its evolution to a stage where its surface temperature is high enough (30,000–35,000 K) to give rise to the observed ionization¹⁰. It has also been suggested¹¹ that G357.7–0.1 is a new type of object in which an energizing event is responsible for the injection of relativistic electrons which emit synchrotron radiation and in which the current activity is at the western edge. According to such a hypothesis, the age of the source is $\sim 5 \times 10^5$ yr and a period of $\sim 25,000$ yr is easily available for the formation of the star at the expanding edge.

There have been suggestions for star formation triggered by the expansion of a SNR in W44 (ref. 12) and W28 (ref. 13). G357.7–0.1 is unique in the respect that it reveals an association between a far-infrared object and a compact radio source. It is of interest that G357.7–0.1 is situated on the edge of an inclined disk of gas that has been postulated to exist in the galactic centre¹⁴. A prominent feature has also been observed in the ^{12}CO ($J=2 \rightarrow 1$) line in the direction of the compact source³. Further high-resolution molecular observations are needed to study the source in greater detail. High-resolution mid-infrared observations to determine the source size in the infrared, and near-infrared observations to reveal the embedded object, as well as a detailed study of the IRAS image data are also needed. Finally, it is important to establish the thermal nature of the compact radio source by making polarization and radio recombination line measurements of the compact source.

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Long period oscillations in solar diameter measurements

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Observations of the Sun with a Danjon-type visual astrolabe have been recorded since 1975 at the CERGA Observatory. The primary goal of these observations is to determine the orbital elements of the Earth around the Sun. However, they also provide very good quality, homogeneous values of the solar diameter. The working principle of the astrolabe, which relies on the method of equal elevations¹, has been described elsewhere². The diameter value is obtained from the difference between the zenith distances of the solar centres corresponding to the instances of successive limbs crossing the same almucantar. The solar diameters are obtained up to 16 times per day. The heliographic latitude of the observed points varies throughout the day and changes with the seasons. In the present study, we have discarded the data taken at zenith distances $> 60^\circ$ because of their lower quality on average. We consider then all the retained measurements on equal footing. The existence of a long period, $\sim 1,000$ days, in the variation of the solar diameter has already been reported³. The low-frequency domain of the power spectrum deserved a more detailed analysis which the more recent data permit. Here, we present this analysis, which reveals several previously undetected periodicities.

Figure 1a shows the low-frequency part of the power spectrum computed from 506 daily averages of the solar diameter measured between 1975 and 1984. These points are scattered over 3,600 days (with fewer observing days in the winters). Data for year 1977 are missing because the astrolabe was not operating then. We use a 16,384 ($=2^{14}$) points fast Fourier transform, so that the spectrum is slightly oversampled. The window function has been calculated and shows a very small bump around

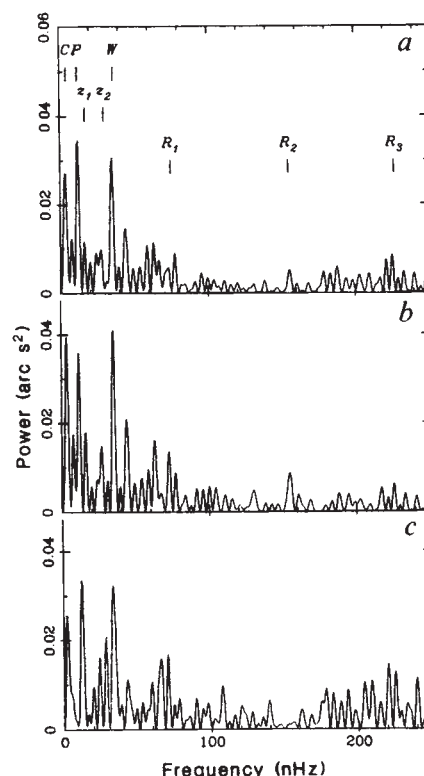


Fig. 1 Power spectra of the solar diameter variations. a, Morning and afternoon data; b, morning data only; c, afternoon data only.

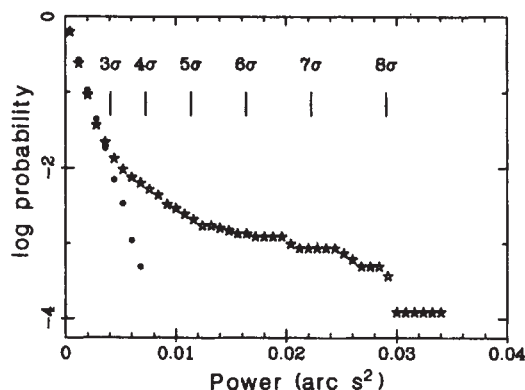


Fig. 2 Statistical distribution of spectral estimates; ●, randomized data; ☆, original data.

frequency $1 \text{ yr}^{-1} = 31.7 \text{ nHz}$. All the peaks discussed here are free from any 31.7 nHz aliasing effect. Important side lobes in the power spectrum are caused by the periodic weekly interruption of the observations, but because we are considering only very long periods, no special pretreatment of the spectrum is necessary. To assess the significance of peaks in the power spectrum, we propose three methods. (1) We divide our data set into two parts: the morning and the afternoon observations. We can then obtain two independent spectra of less quality (Fig. 1b,c). Figure 1 shows that the three major low frequency peaks C, P and W at least, are qualitatively significant.

(2) A more quantitative analysis relying on a study of the statistical distribution of spectral estimates, has been used previously in discussing solar gravity modes^{4,5}. Figure 2 shows the logarithm of the probability of spectral estimates $p(w)$ with size larger than a given value w plotted against that value. The stars correspond to the observed spectrum. The dots are from a similar spectrum computed with the same data taken at random and distributed on the actual observing days, thus preserving the same window and noise characteristics but destroying the coherent signal. Pure independent gaussian noise distributions with variance σ^2 in the real and imaginary part of the Fourier transform would yield a power spectrum described by

$$p(w) = \exp\left(-\frac{w}{2\sigma^2}\right) \quad (1)$$

In our logarithmic representation, such a noise would show up as a straight line whose slope is a measure of the variance σ^2 of the distribution of the Fourier components. One can observe on Fig. 2 that the actual distribution of the randomized data spectrum is not significantly different from a straight line. On the contrary, for the real data spectrum, one sees an excess of large amplitude estimates indicating the existence of peaks well above the noise level. The slope of the distribution for small values, or a direct calculation allows the variance to be measured.

$$\sigma^2 = 4.6 \times 10^{-4} \text{ arc s}^2 \quad (2)$$

Table 1 gives the frequencies of some selected peaks and their amplitudes.

(3) One may expect that magnetic activity, such as sunspots, plagues or filaments, will modify the visual definition of the solar limb, or will be connected to the solar diameter. Thus, it would be appropriate to compare our spectrum with the spectrum of some global solar activity index, for example, the daily Zürich Sunspot Number (ZSN) during the same period. Wolff⁶ has already published a spectrum of more than 200 yr of ZSN computed from monthly averages. Using a similar method, we first eliminate the very low frequency part of the spectrum by subtracting from each daily point a running mean over 2 yr centred on this point; this provides the spectrum in Fig. 3. Some peaks in this spectrum coincide with peaks in the solar diameter spectrum, particularly, the major peak around 36 nHz (320 days), our W mode. This particular frequency has already been

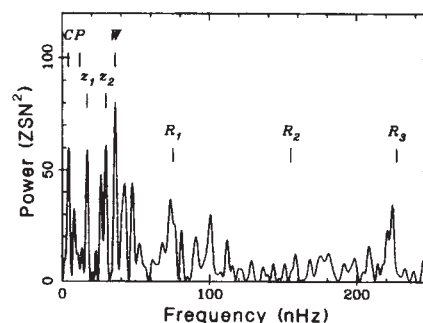


Fig. 3 Power spectrum of the modified ZSN.

mentioned by Wolff. Indeed, his 200 yr spectrum was dominated by a line located at precisely 36.3 nHz. Next, in our ZNS spectrum, we identify two peaks, z_1, z_2 . The first one, z_1 , also appears in the diameter spectrum, but z_2 does not.

A further solar activity frequency analysis has been produced by Rieger *et al.*⁷ from the occurrence of high energy flares detected by the solar maximum mission and GOES satellites. Rieger *et al.*⁷ identify a main frequency at 75 nHz and two minor modes at 155 and 227 nHz. (These three frequencies are denoted R_1, R_2, R_3 in Figs 1 and 3.) The two minor modes correspond with well defined peaks in the ZSN spectrum, and with peaks standing over the 4σ level in the solar diameter spectrum. We conclude that because some of the features are shared by both spectra we are dealing with intrinsic solar frequencies.

These three approaches permit us to assess the reality of the main modes that we have found in the low-frequency part of the solar diameter power spectrum, before discussing their physical significance. Table 1 includes all the frequencies that have been identified in the occurrence of solar flares by Rieger *et al.*⁷, in the solar diameter and in the ZSN spectra by us.

With the exception of the major peak P, the solar diameter signal seems to be correlated with the ZSN. Figure 4 shows the cross-correlation function

$$f(d) = \frac{\langle \varphi(t) \times w(t+d) \rangle}{\sqrt{\langle \varphi^2 \rangle \langle w^2 \rangle}} \quad (3)$$

where φ is the solar diameter and w is the ZSN, both of them being shifted so that their mean values are set to zero and d is the time offset in days between diameter and magnetic observations. In our convention, a positive value of d means that we correlate ZSN values with solar diameter values occurring d days earlier. In fig. 4, we have used the raw ZSN. We observe an anti-correlation which shows that an active Sun has a smaller diameter. This effect can be compared with the diminution of the characteristic horizontal scale of granules during solar activity maximum⁸. In both cases, it looks as though solar activity was triggering a shrinking of granules, horizontally as well as vertically, of the order of 200 km. The maximum anti-correlation occurs roughly at $d \approx 200$ days as though active regions were, on average, depressing the limb more 200 days before their maximum development. The first peak, C, may now be easily

Table 1 Low-frequency peaks observed in solar diameter and in solar activity 1975-84

Label	Frequency (nHz)	Amplitude (arc s)	σ level	Other determinations (nHz)
C	3.3 ± 1.5	0.16	7.7	4.4 ± 1.5 (Z)
P	11.9 ± 1.5	0.18	8.7	
z_1	16.2 ± 1.5	0.10	5.0	16.8 ± 1.5 (Z)
z_2				29.5 ± 1.5 (Z)
W	35.0 ± 1.5	0.17	8.1	36.0 ± 1.5 (Z)
R_1	77.3 ± 1.5	0.09	4.4	75 (R) 73.4 ± 1.5 (Z)
R_2				155 (R)
R_3	225.3 ± 1.5	0.09	4.3	227 (R) 224.2 ± 1.5 (Z)

Z, from ZSN, R, after Rieger *et al.*⁷.

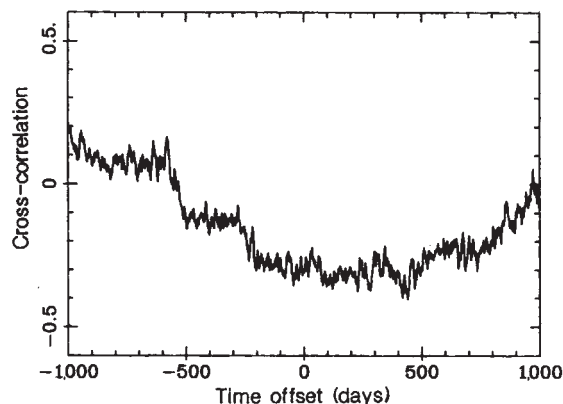


Fig. 4 Cross-correlation between the solar diameter and the ZSN.

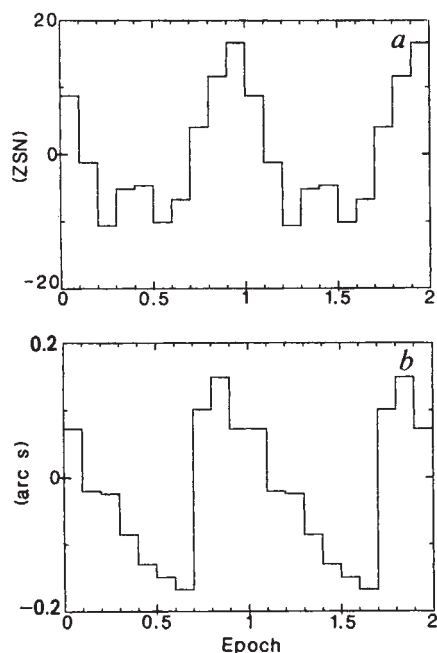


Fig. 5 Superposed epoch analysis at 320 days of the ZSN (a) and of the solar diameter (b).

interpreted, even if it is very close to the lowest possible frequency that can be significantly deduced from our finite samples and is a signature of the solar 11-yr cycle for the ZSN. A longer time series would make the peak sharper around the frequency (solar cycle)⁻¹. With respect to the diameter, our time series does not permit us to distinguish an 11-yr periodicity from another slow variation. However, the trend of the raw data favours the first hypothesis. Furthermore, the phases of the Fourier components of φ and w are very nearly opposite in that frequency range, thus confirming the long-term anti-correlation between diameter and activity.

We come now to mode W which dominates the ZSN spectrum. It has a period of the order of 320 days. To clarify the relationship between the diameter and magnetic oscillations, Fig. 5 shows the superposed epoch variations of both sets of observations. The same time origin has been used in both cases. The 320-day period appears clearly in both structures. Note that the maxima appear close to each other, indicating that the two oscillations are nearly in phase. A fine analysis of the complex Fourier spectra yields the time offset between them: $d_{\text{offset}} \approx 20$ days. If the appearance of sunspots is the cause, and the solar diameter variation the effect, it looks as if the solar diameter would rise sharply in the early days of the spots, and then more slowly decrease after their decay. This could be related to radiation blocking by sunspots which causes the surrounding surface to rise up momentarily (as suggested by E. Fossat).

Peak P , is the dominant feature of the solar diameter variation, standing out at the 8.7σ level. Its period is of the order of 1,000 days; it is neither detectable in our ZSN spectrum nor in Wolff's analysis⁶ and seems to have no counterpart in solar flares. Similar periodicities have, however, been detected in $\text{Ca}^+ \text{K}$ plage areas⁹ and in λ 10-cm solar flux¹⁰. One is thus tempted to assign to it a deeper origin inside the solar body and to link it with the marginally significant periods found in the variation of the solar neutrino flux^{11,12}. Another explanation which has been proposed by Wolff⁶, could be found in global long-period solar oscillations caused by interferences of internal gravity modes. But it would still be necessary to explain why some of these interferences show up in Wolff's ZSN spectrum, whereas peak P shows up only in the diameter spectrum.

In summary, solar diameter variations as observed along a solar cycle reveal long period oscillations of characteristic amplitude 0.1 arc s. The physical origin of these variation is found in deep layers of the solar body, possibly in the core for the 1,000 days oscillation. Because some of these modes are also found in the ZSN, we think that an harmonic analysis of more detailed activity indexes may also prove very fruitful. Our solar diameter data also contain information about the heliographic latitude at which the measurements are made. We have not yet used this information. The recent increase in the number of observations performed every day, and the automation future of the instrument will provide us with a very efficient duty cycle. A comparison between our solar diameter fluctuations and SMM/ACRIM radiometer measurements is in preparation. Unfortunately, we are still far from a full solar cycle coverage of irradiance observations. This continuous monitoring from space is highly desirable.

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Geometric and electronic structure of a semiconductor superlattice

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Esaki and Tsu¹ have proposed that a semiconductor superlattice (a periodically repeated arrangement of alternating layers of two III–V semiconductors) might exhibit negative differential mobility (NDM) if electrons could be injected into regions of negative effective mass in the band-structure created by the superlattice. A weak NDM has subsequently been observed², but nothing more has been reported since then. We have recently seen strong NDM in a 21-period AlGaAs/GaAs superlattice³. The current-voltage (I – V) characteristics show fewer, but sharper, features than reported by Esaki and Chang². Here we report the correlations between (1) the design and predicted performance of an ideal semiconductor superlattice and (2) the structure and actual performance of the 21-period superlattice. Calculations of the energy

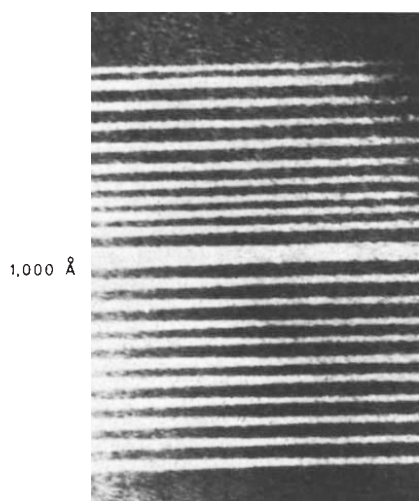


Fig. 1 Dark-field electron micrograph of a 21-period superlattice.

dependence of electron transmission through the ideal and the actual superlattice show gross band-structure features at similar energies. Fine structure in electron transmission through the actual superlattice may be the origin of fine structure in the measured I - V characteristics. The results imply a certain tolerance of the electronic structure towards modest fluctuations in the superlattice parameters, which may be of importance in the realization of superlattice devices.

The superlattice³ was grown in a Vacuum Generators V80H MBE machine, each period consisting (ideally) of 3 nm of $\text{Al}_{0.25}\text{Ga}_{0.75}\text{As}$ and 6 nm of GaAs. The superlattice was grown between thicker layers of heavily doped GaAs which provide ohmic contacts. Figure 1 shows a (002) dark-field electron micrograph of a thin cross-section of the superlattice, recorded on a JEOL 200CX electron microscope. The darker regions are GaAs, with the lighter regions being $\text{Al}_x\text{Ga}_{1-x}\text{As}$. Detailed modelling is required if the x -value and any variations in x from layer to layer are to be extracted from the degree of contrast, but $x = 0.25$ is not inconsistent with the data. Apart from the anomalously thick central AlGaAs layer, there are fluctuations in the remaining layers. The thickness of each layer has been measured directly from high-resolution lattice images⁴ which yield a mean thickness (and r.m.s. deviation) of 52 (10) Å for the GaAs layers and 33 (5) Å for the AlGaAs layers. The layer thicknesses seem quite uniform over lateral distances that are large relative to the superlattice thickness.

We have calculated the quantum-mechanical transmission amplitudes, at zero electric field, of electrons (as a function of their energy above the GaAs conduction band edge) tunnelling through both the ideal and actual superlattice⁵. The $\text{Al}_x\text{Ga}_{1-x}\text{As}$ layers act as tunnelling barriers whose energy height is given by the conduction band offset between the two materials. This offset is a matter of active debate (see ref. 6 and references therein); 70% of the total bandgap difference has been used here. The results for a one-dimensional model of the superlattice are shown in Fig. 2. The energy ranges for resonant transmission (which corresponds to allowed energy bands) are broadly similar in the two examples. The disorder in the actual superlattice reduces the effectiveness of transmission in the lowest band. In a further development of the theory, the reduction would be modelled by a reduced effective conductance⁷. The disorder also produces sharper structure, particularly on the higher energy side of the first band of resonant transmission.

Figure 3 shows a typical low-temperature I - V characteristic of the superlattice. At low bias the behaviour is ohmic; the doping conditions were chosen to ensure that the Fermi level is in the centre of the first superlattice band. The most notable features are the instabilities at which the voltage switches between two values. We also show the featureless I - V characteristic of a two-period 'superlattice' used as a control to monitor

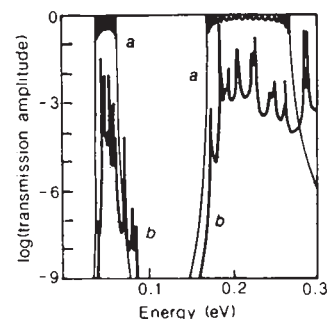


Fig. 2 The energy dependence of transmission amplitude of electrons through an ideal 21-period superlattice (trace a, light line) compared with that of the actual superlattice (trace b, heavy line).

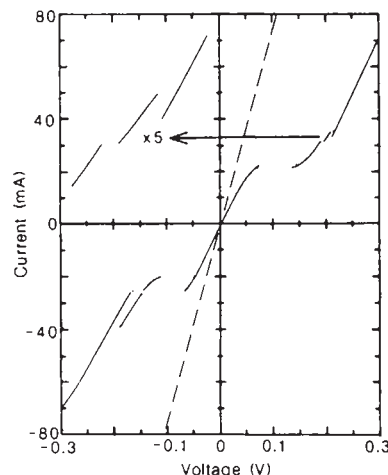


Fig. 3 I - V characteristics of a 21-period superlattice (full line, with discontinuities shown dotted) and a 2-period control structure (broken line). The temperature was 77 K and the device area was $8 \times 10^{-9} \text{ m}^2$. A region of fine structure is shown enlarged (arrowed).

contact and series resistances. Allowing for these resistances, the onset of the major instability occurs when the voltage drop across the superlattice equals the width of the first superlattice band. Moreover, the size of the voltage discontinuity equals the separation between the first and second superlattice bands. A detailed explanation of this, together with supporting evidence, is given elsewhere³. We believe that the additional fine structure is associated with sharp resonances in the transmission coefficient for the first superlattice band, but our ability to associate gross features in the I - V data with the superlattice band-structure is not affected by the irregularities in the actual superlattice. Although our calculations show that the thick barrier does not significantly modify the transmission coefficient, it forms a natural nucleation centre for the high field domain intrinsic to NDM device operation³. We believe this to be the first such correlation of properties between an idealized and an actual superlattice.

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Muertos Trough subduction—microplate tectonics in the northern Caribbean?

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The relative plate motion between the North American and Caribbean plates has been commonly assumed to take place in the form of oblique subduction along the Puerto Rico Trench¹. West of Hispaniola this plate boundary becomes pure strike-slip. Nevertheless, it has been suggested that this boundary is more diffuse, with part of the plate motion taken up by subduction along the Muertos Trough (MT) south of Hispaniola²⁻⁵ (Fig. 1), but there has been no direct seismological evidence of subduction to confirm this. Here we present the results of a study of the 24 June 1984 earthquake beneath MT. The focal mechanism shows thrusting at a depth of 32 km, which together with reports of a great earthquake in 1751 confirms that MT is an active plate boundary. This implies that plate motion is accommodated by several tectonic boundaries delineating microplates caught between the Caribbean and North American plates (Fig. 4).

Subduction along MT has been suggested on the basis of multichannel reflection lines (inset of Fig. 2) obtained by Ladd and others². On these profiles, the basement of the Caribbean plate can be traced for at least 40 km to the north of MT, dipping at an average of 11°. Additional evidence for subduction along MT consists of a 100-mGal negative free air gravity anomaly centred over the trough, which suggests that the trench is not in isostatic equilibrium⁶. The lack of turbidites on the Venezuelan Basin, to the south of MT³, suggests that MT has existed at least since the late Cretaceous. Biju-Duval finds further

evidence for the subduction of Caribbean crust in the geology of southern Hispaniola⁴.

Some workers have argued that MT subduction ceased by Oligocene⁷ or late Eocene time⁸. However, many earthquakes between the southern coast of Hispaniola and MT are too far south and too shallow to be directly associated with subduction along the Puerto Rico Trench to the north, strongly suggesting that they are associated with an active tectonic boundary along MT (Fig. 1). Furthermore, there is historical evidence for earthquakes to the south of Puerto Rico and Hispaniola. For example, reports of the 18 October 1751 earthquake (mag ~8) show extensive damage only to the south of Hispaniola, which strongly suggests that the earthquake occurred along the southern margin and not along the Puerto Rico Trench to the north.

The earthquake of 24 June 1984 (6.7 Ms), studied here, occurred south of Hispaniola beneath the San Pedro Basin (Fig. 1). Long-period P and SH waveforms were inverted to determine the source mechanism, focal depth, scalar seismic moment, and the duration of the source time function. This technique is used to calculate the variance between the observed and synthetic waveforms and, through an iterative process, to minimize the errors until convergence criteria are reached⁹. The data used are digitally recorded seismograms from the Global Digital Seismic Network with a high signal-to-noise ratio. A few analog seismograms were digitized manually to improve the azimuthal coverage. The results of the inversion (Fig. 3) show a thrust mechanism at a focal depth of ~32 km with one nodal plane shallowly dipping (~10°) to the north. Synthetic seismograms for this solution are shown in Fig. 3.

Some of the other earthquakes south of Hispaniola, for which we have obtained a preliminary estimate of the focal depths and source mechanisms, are deeper than the shallow thrust earthquake of 24 June; these events occur within the Caribbean plate and their P axes consistently plunge shallowly to the north (Fig. 2), indicating that the Caribbean plate deforms under down-dip compression. This may result from the resistance encountered in subducting a plate with thick buoyant crust^{10,11}, or from the deformation associated with the bending of the

Fig. 1 General bathymetry and seismicity of the Hispaniola region. Although most of the shallow seismicity (depth ≤ 70 km, ●) occurs along the Puerto Rico Trench north of Hispaniola and Puerto Rico, shallow seismicity is observed along the inner wall of the Muertos Trough. The 24 June 1984 earthquake (large circle) is the largest of these events. The track of line VB-2N (see Fig. 2) is shown. Bathymetric contours are in metres; epicentres are from various sources: 1950-63 (ref. 1), 1964-81 (International Seismological Centre, Newbury, Berkshire, UK), 1981-84 (National Earthquake Information Center, US Geological Survey, Denver, Colorado). Open symbols are intermediate-depth events (depth ≥ 70 km).

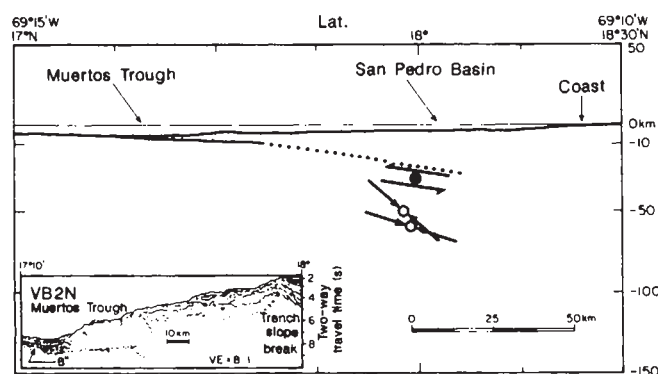
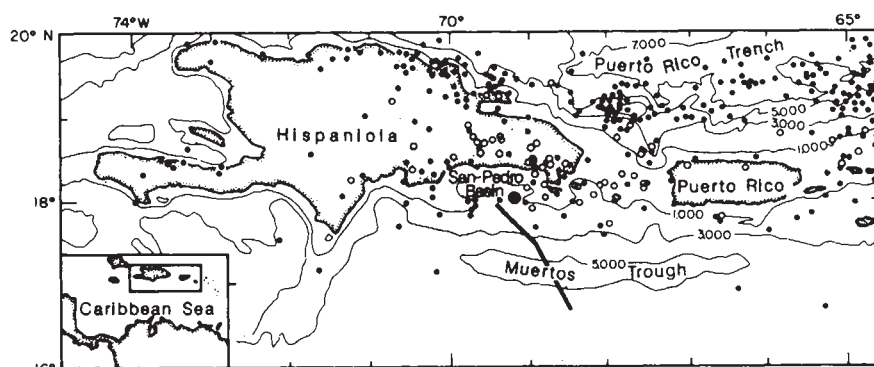


Fig. 2 Cross-section through the southern margin of Hispaniola. Solid circle with opposing arrows shows location and sense of motion of the 24 June 1984 earthquake. Open circles indicate locations of previous events; arrows show direction of maximum compression. These events taken together suggest active thrusting between the Caribbean plate and the southern coast of Hispaniola, and compressional deformation of the subducted Caribbean plate. Inset, a line drawing of the multichannel reflection profile from Ladd and others². B" is the reflecting horizon traceable across Caribbean and VE, the vertical exaggeration, is eight to one. Note the deformation of sediments along the inner wall of the trench, and the continuation of the Caribbean crust reflector for 40 km landward from the trough axis; the dotted line in the main figure is a downward continuation of this reflector.

Fig. 3 Results of the P and Sh body-wave inversion of the 24 June 1984 earthquake (18.01° N, 69.20° W). Depth = 32 km, seismic moment 4.2×10^{25} dyn cm. *a*, The focal mechanism from P waves. This is a lower hemisphere projection; closed symbols are compressional first motions and open symbols are dilatational first motions. The best-fit strike is 97° , dip is 81° and slip is 92° . Some representative waveforms are shown: solid traces are the observed wave, and dashed traces are computed. *b*, The equivalent solution for SH body-waves. The three-letter code refers to the seismic station.

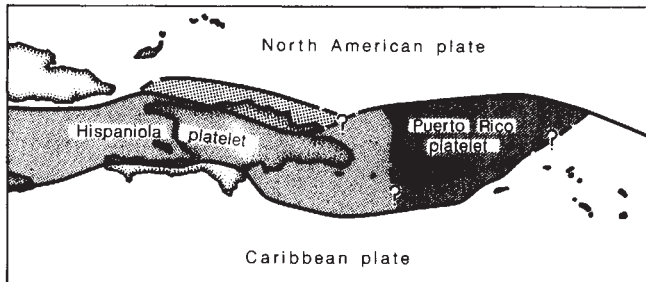
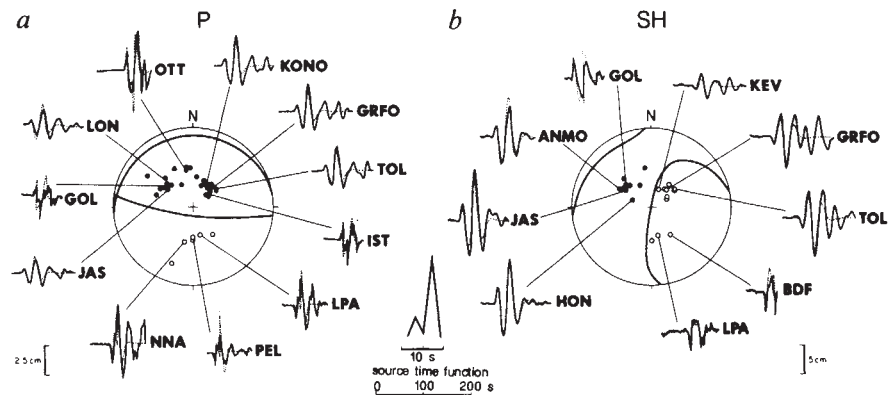


Fig. 4 Sketch of the possible platelet boundaries along the North American-Caribbean plate boundary. Platelet margins are deduced from historic and recent seismicity, marine reflection records, and geological mapping.

subducted slab¹². The lack of a well-developed Benioff zone and the absence of Neogene volcanics may be due to the low rate of underthrusting.

Active subduction along MT implies that an active plate boundary runs south of Hispaniola and possibly Puerto Rico. Thus, the northern Caribbean appears to be composed of one or more microplates sitting astride two larger plates in a manner similar to that of the Mediterranean¹³. The boundaries of the microplates may be tentatively defined on the basis of recent seismicity, historic earthquakes and sediment deformation observed in seismic profiles. To the west of 71° W, where the southern plate boundary appears to continue on land⁴, earthquakes are shallow and less frequent. McCann and Sykes¹⁴ suggest that subduction is inhibited here by the thick lithosphere of the Beata Ridge to the south, and the Bahamas Platform to the north. Thus, the transition from a thrust plate boundary (north and south) to the pure left-lateral strike-slip motion observed in Haiti¹⁵ appears to occur near 71° W. Recent seismic activity and historical reports of moderate-sized earthquakes to the south of Puerto Rico suggest that MT is active until it dies out near the Virgin Islands (Fig. 1). Shallow seismicity along the Anegada Passage probably represents the easternmost boundary of the Greater Antilles microplate system (Fig. 4)¹⁶. If one uses a relative rate of plate motion of 3.5 cm yr^{-1} between the Caribbean and North American plates¹⁴, the higher seismicity along the Puerto Rico Trench places an upper limit on the potential rate of motion along MT of 2 cm yr^{-1} . A more realistic rate is probably of the order of 1 cm yr^{-1} .

The shallow events that occur between Hispaniola and Puerto Rico suggest that these islands lie on two separate platelets (Fig. 4). The lack of crustal seismicity in Puerto Rico and the Virgin Islands observed by the seismic networks there^{17,18} indicates that those areas constitute a single platelet (Fig. 4). The complex geology and seismicity of Hispaniola suggest that this island may be split into smaller terranes; the most obvious example of this is the Septentrional Fault in northern Hispaniola that separates the Cordillera Septentrional from Hispaniola proper¹⁹ (Fig. 4).

Thus, the 24 June 1984 earthquake shows that strong earthquakes do occur along a subduction zone off southern Hispaniola, and the 1751 earthquake indicates that the 1984 event is not unique. This implies that the seismic hazard here may be higher than has been assumed previously. An inversion of the P and SH body-waves from the 1984 earthquake yields a focal mechanism solution that is consistent with subduction of the Caribbean plate to the north beneath Hispaniola. This suggests that eastern Hispaniola and Puerto Rico have been severed from the Caribbean plate and sit as platelets, being underthrust simultaneously by both the Caribbean and North American plates.

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Seismic stratigraphy of the flexural moat flanking the Hawaiian Islands

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The elastic thickness of oceanic lithosphere, based on flexure studies in the region of surface loads, is two to three times smaller than its seismic thickness¹. Thus, as a load ages, the thickness of the mechanically supportive part of the lithosphere must decrease from its short-term (seismic?) thickness to its long-term mechanical thickness. Extrapolation of data from experimental rock mechanics suggests that thinning occurs by brittle failure in the uppermost part of the lithosphere and thermally activated creep in its lower part². Modelling shows that for load ages less

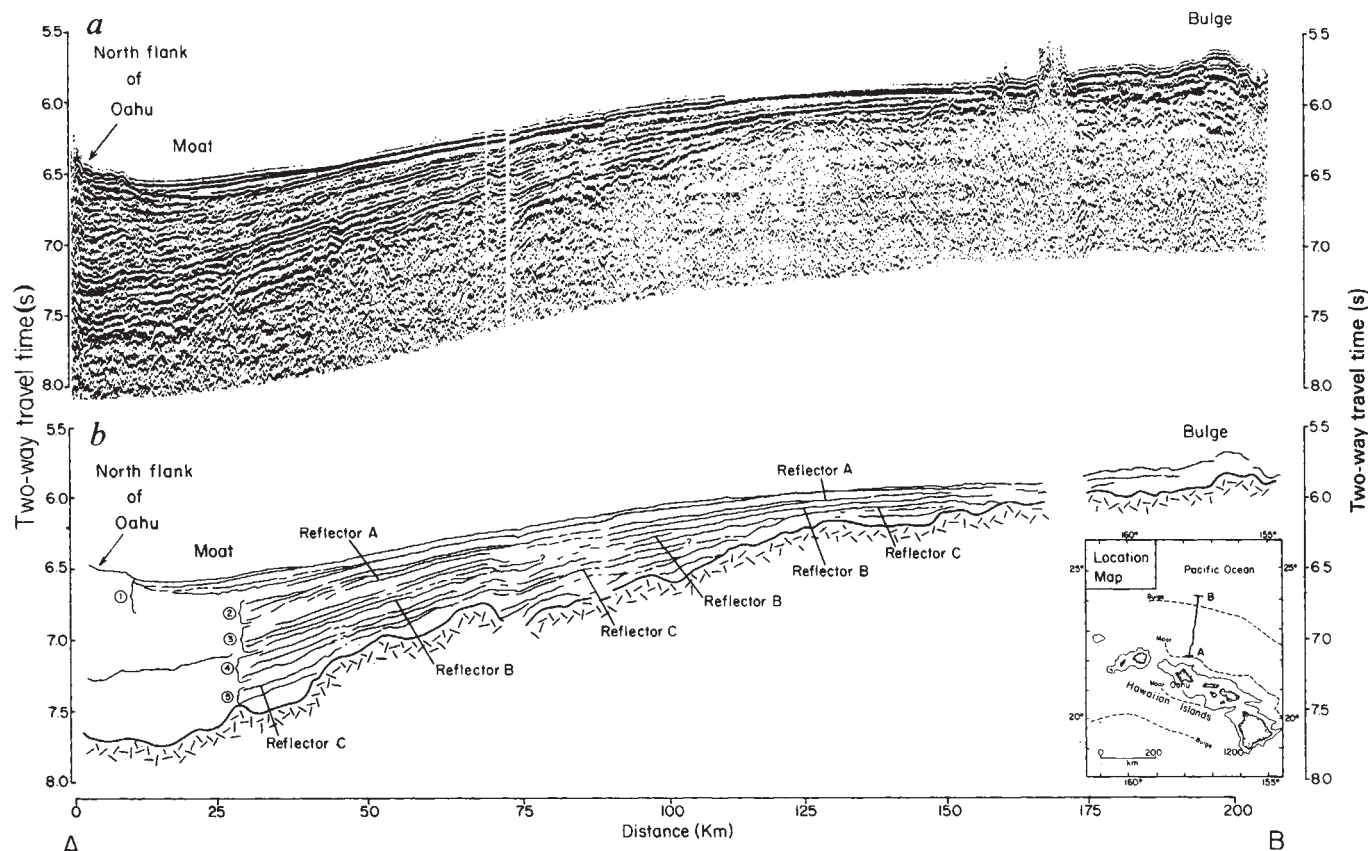


Fig. 1 Multichannel seismic reflection profile AB of the flexural moat and bulge north of Oahu, Hawaii. *a*, Stacked common mid-point record obtained by RV *Conrad* during August 1982. The receiver consisted of 48 groups of hydrophones on a 3.6-km-long streamer towed behind the ship. The sound source was an airgun array with a total capacity of 2,500 cubic inches. *b*, Line drawing of the stacked record. The numbers represent the units described in the text. The heavy line with hatching indicates the upper surface of the oceanic basement.

than about 1 Myr, flexural moats change shape and the peripheral bulges migrate in towards the load³. These transient changes in flexure are best manifest, we believe, in the stratigraphical sequences that infill flexural moats. The largest thicknesses of sediments occur in moats flanking orogenic belts^{4,5}. However, the stratigraphical record in these basins is complicated by the effects of compaction, sub-aerial erosion and sea-level changes, and by the fact that the loading history extends over very long periods of time. Here we describe a multichannel seismic reflection profile across a flexural moat flanking the volcanic load of Oahu in the Hawaiian Islands. The advantages of Oahu are that it is located in the interior of a plate, the load is 2.7 Myr old and formed within 1 Myr⁶, and the flexural moat was not significantly affected by sea-level changes and erosion. We show that the observed stratigraphical patterns cannot be explained by changes in material influx, load distribution, and rapid relaxation of the lithosphere to its long-term mechanical thickness. Rather, the observed patterns suggest a prolonged relaxation (>1 Myr) due to either local or regional re-heating of the lithosphere. This re-heating has important implications for the relationship between elastic thickness and age of the lithosphere at the time of loadings.

Figure 1 shows common mid-point (CMP) multichannel seismic reflection line 314 of the flexural moat NNE of Oahu, Hawaii. Individual traces were demuxed, gathered and stacked in 50-m bins⁷. Predictive deconvolution was applied after stacking and the data were bandpass filtered (6–30 Hz). Although only one CMP display is shown in Fig. 1, other types of display have also been considered, such as near-trace plots and variable horizontal exaggeration.

The main feature shown in Fig. 1 is a poorly to well stratified wedge of material which infills the region between the northern flank of Oahu and the peripheral bulge. The upper surface of the wedge (the sea floor) is tilted by about 0.75 s from the crest of the bulge to the moat, corresponding to an increase of about

580 m in sea-floor depth. The lower surface is defined by a tilted discontinuous diffractive reflector, similar in character to reflectors associated with the top of the oceanic crust⁸.

Seismic refraction data in the region^{7,9} show that the average P-wave velocities in the wedge range from 3.5 to 4.4 km s⁻¹. These velocities imply that the wedge thickens to about 2.2 km beneath the flank of the island. The cross-sectional area of the wedge out to a distance of 160 km from the flank of the island is about 157 km², which is 5–10 times larger than would be expected over a similar distance in the deep Pacific ocean basin. Thus, the material comprising the wedge was probably derived from the island and is volcanoclastic in origin. If all the material in the cross-section in Fig. 1 was eroded from Oahu, then its average elevation must have stood about 3–4 km above its present height.

The general reflector configuration within the wedge is divergent towards the island. Individual reflectors can be followed laterally for several tens of kilometres, although they often exhibit a hummocky or even broken characteristic. Near the island, reflectors are discontinuous and cannot be traced laterally. Additionally, a velocity inversion analysis⁷ shows a high-velocity cap (4.5–5.4 km s⁻¹) at 6.7–7.2 s two-way travel time, suggesting that a basaltic sill or volcanic flow may have been emplaced within the moat infill. In the wedge, seismic reflection patterns of the type normally associated with erosional surfaces¹⁰ do not appear to be present. However, many reflector terminations can be identified in the wedge and are spread out over most of its length.

For purposes of description, we have divided the seismic stratigraphy of the wedge into five principal units (Fig. 1*b*). Each unit is bounded by a high-amplitude reflector and shows a distinct pattern within it. Unit 5 drapes and infills rough topography of the upper surface of the oceanic crust. It is probable therefore that this unit pre-dates the formation of the

flexural moat by the Hawaiian volcanic loads. Unit 4 is bounded at its base by a discontinuous reflector (C, Fig. 1b) and at its top by a high-amplitude reflector (B). Some of the reflectors in unit 4 appear to overstep progressively towards the bulge, although the resolution in this deep unit is poor. The next unit above, 3, is capped by another high-amplitude reflector (A) and clearly shows progressive overstepping from 75 to 160 km. This pattern is reversed in unit 2 in which most of the reflectors offstep progressively towards the islands. The uppermost unit, 1, does not show a preferential direction of overstepping or offstepping. Furthermore, the infilling material 'ponds' close to the flank of the island, with reflector termination at both ends. Hence, the overall stratigraphical pattern is one of migration of reflector terminations towards the bulge in the lower part of the section to migration towards the flank of the island in the upper part, and a random pattern of migration and ponding in the top-most part of the moat infill. We caution, however, that the distance between individual reflectors is close to the limits of the seismic resolution and therefore the exact location and relationship at reflector terminations are not always easy to determine.

We suggest that three principal factors have controlled the reflector patterns in the wedge flanking Oahu: the rate of material influx, temporal and spatial changes in the distribution of the Oahu load, and tectonic movements of the underlying oceanic lithosphere. In order to investigate the role of these different factors, synthetic stratigraphical models have been constructed and compared with the observed seismic section. In the absence of age control, we assume that individual reflectors correspond to chrono-stratigraphical horizons.

A commonly used rheology of the oceanic lithosphere is the yield stress envelope (YSE) model². In this model, the strength of oceanic lithosphere is limited by brittle failure in the form of Byerlee's law in the upper part of the lithosphere and by ductile flow in the form of the power law ($n=3$) and the Dorn law in the lower part.

Figure 2a shows the relaxation of a two-dimensional elastic plate caused by a distributed load the size of Oahu. The strength of the plate is limited by the YSE, and the elastic thickness varies laterally because of flexure-induced stress concentrations. We describe the relaxation process in six time steps of 0.5 Myr duration, which sums to the approximate age of Oahu⁶. Within each time step, the flexure was calculated by loading a thin elastic plate and iterating until convergence was achieved for the corresponding YSE. The strain rate in the ductile flow law was computed for each time step assuming the empirical relationship³ between strain and time. A synthetic stratigraphy was constructed by successively adding the differences of individual flexure curves. At each time step, we assumed that the material filled the flexural moat was deposited horizontally and that it filled to the pre-deformational surface. A comparison between Fig. 1 and Fig. 2a shows poor agreement between the synthetic and the observed section.

In order to fit the observed section better, the parameters in the simple model shown in Fig. 2a were varied. We first investigated changes in the material influx to the moat by varying the level of infill for each time step. We assumed a decreasing rate of material influx—consistent with the decrease in average elevation of the Hawaiian Islands with time. Figure 2b shows an example where infill level rises with time in such a way that the final height of the infill is 370 m below the pre-deformational surface. A limit to the moat infill is in accord with the observed differences in sea-floor depths between the bulge and the moat (Fig. 1). The stratigraphical pattern produced in the model in Fig. 2b is one of progressive overstepping towards the bulge and little or no divergence of individual horizons towards the load. Other variations in the rate of material influx produced similar stratigraphical patterns. Decreasing the six time steps to 0.075 Myr (total of 0.45 Myr.) did not alter the stratigraphical pattern significantly. Any further reduction in the time step would imply an unreasonably high accumulation rate in the moat. A comparison of Fig. 2b with the observed section shows

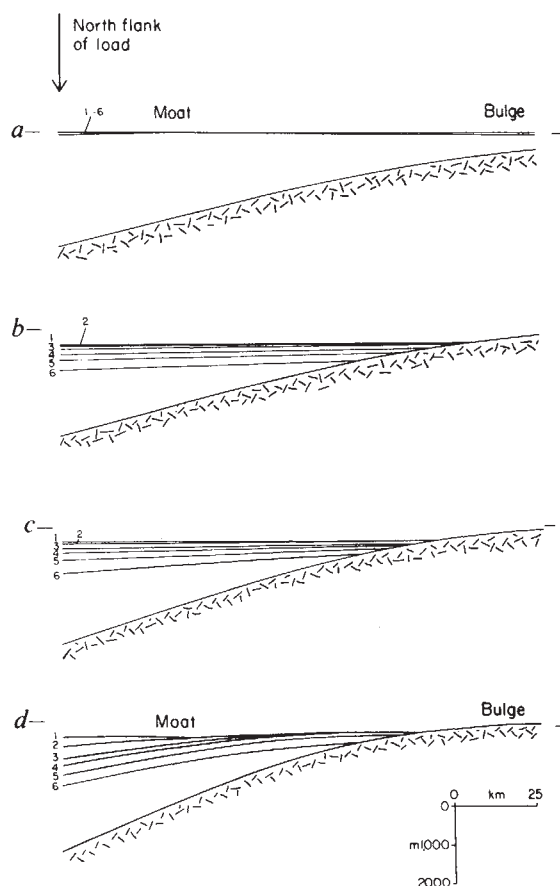


Fig. 2 Synthetic stratigraphy predicted by a two-dimensional continuous elastic plate model loaded by a load the size of Oahu. The strength of the plate is limited by the YSE and the relaxation process is described in six time steps. *a*, Infill to pre-deformational surface. Time step, 0.5 Myr. Ductile flow given by the power law and Dorn law with activation energy = 510 kJ mol⁻¹ and other parameters as defined by Bodine *et al.*³. *b*, Same as *a* except for infill to a variable level. *c*, Same as *b* except that ductile flow is given by temperature-dependent newtonian viscosity with activation energy = 170 kJ mol⁻¹ and basal viscosity of 10¹⁸–10²⁰ Pa s (ref. 15). When expressed in power law form, the parameters are stress power = 1 and pre-stress term = 1.16 × 10⁻⁸–1.16 × 10⁻¹⁰ bar⁻¹ s⁻¹. *d*, Same as *b* except that the equivalent thermal age of the lithosphere is reduced through time. The parameters used to produce the models are given in Fig. 5a of Watts *et al.*⁹ for the load size, load density, infill and mantle density.

that while a variable-infill model could explain the pattern of overstepping shown in units 1 and 2, it cannot account for either the patterns in the upper units or the overall tilting of individual reflectors.

The synthetic stratigraphy shown in Fig. 2b could not be significantly altered by changing the width of the load or the form of the ductile flow law. An increase in the load width, for example, would only enhance the pattern of overstepping seen in Fig. 2b. Moreover, the use of a temperature-dependent newtonian viscosity expressed as a power law does not significantly alter the overall pattern in the moat (Fig. 2c).

We found that the stratigraphical patterns observed in the moat could only be explained by a model in which there was a further reduction in plate rigidity with time. This reduction was achieved by perturbing the oceanic geotherm and hence altering the depth at which material responds to loads by thermally activated creep. Figure 2d shows a flexure model with similar parameters as in the model in Fig. 2b except for the choice of the oceanic geotherm. In Fig. 2b, the geotherm is set to correspond to that of 80-Myr-old sea floor and does not change with time. In Fig. 2d, the geotherm is the same for the first two steps

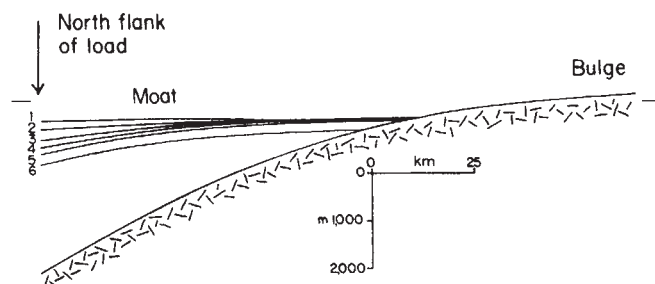


Fig. 3 Synthetic stratigraphy produced by using a thin-elastic plate model and varying the rigidity of the plate through time. Load, time steps and other parameters are as in Fig. 2. In the model, the rigidity is decreased from a normal value of 5×10^{30} (elastic thickness, $T_e = 30$ km) to a minimum of 10^{29} dyn cm ($T_e = 5$ km) beneath the load. The elastic thickness is decreased linearly towards the centre of the load and the width of the weakened zone increases with time step to a maximum value 100 km either side of the load.

of loading as in Fig. 2b, and is subsequently progressively elevated corresponding to a decrease in effective thermal age with time. At the final step of loading, the geotherm corresponds to about 44-Myr-old sea floor.

In contrast to the previously discussed models, the model in Fig. 2d compares well with the observed stratigraphical cross-section in Fig. 1. Specifically, the model shows a divergent pattern of horizons toward the load, a tilted sea floor, and stratigraphical patterns which closely resemble the succession of units in Fig. 1.

The observed section could also be simulated using a thin continuous elastic plate model and progressively decreasing its rigidity over successively wider areas with each load step. The resulting synthetic stratigraphy (Fig. 3) compares reasonably well with the observed profile. We found the model pattern, however, to be extremely sensitive to the spatial and temporal changes in the rigidities that were assumed.

Figures 2d and 3 show that in order to explain the observed stratigraphical patterns, it is necessary to weaken the plate progressively over a longer period of time (~ 1 Myr or more) than would be predicted by the YSE. According to the YSE, the greatest reduction in rigidity occurs over relatively short times ($\sim 10^4$ – 10^5 yr). Moreover, the rate of subsidence is small after 10^4 – 10^5 yr, producing stratigraphical patterns of the type shown in Fig. 2a–c. In order to account for the divergent reflectors in the observed profile, a higher rate of subsidence is required for longer times (~ 1 Myr). In the model in Fig. 2d, the subsidence rate at the edge of the load is persistent at about 0.20 mm yr^{-1} for the past 2.5 Myr. Beneath the load, the rate increases from 0.25 to 1.00 mm yr^{-1} during the same period. Although Moore¹¹ suggested, on the basis of tide-gauge records, that Oahu is stable relative to the west coast of the United States, Gregory and Kroenke¹² pointed out the existence of carbonate reefs on the southern shelf of Oahu whose depth (> 500 m) could not be explained by eustatic sea-level change.

A possible explanation for a sustained weakening of the lithosphere is re-heating and thinning of the lithosphere in the vicinity of the Hawaiian 'hot-spot'. Detrick and Crough¹³ suggested, on the basis of bathymetric data, that 80-Myr-old oceanic lithosphere in the vicinity of Hawaii was re-set over a broad region to a thermal age of 30 Myr in about 5 Myr following the initial heating event. The model in Fig. 2d, in which the geotherm is perturbed to an equivalent age of 44 Myr after about 3 Myr, is in accord with this suggestion. It may also be possible, as Fig. 3 shows, to explain the observed section by a progressive broadening of a zone of weakness in the lithosphere beneath the load. This could have resulted from continued penetration of intrusive material into the portion of the plate that behaves elastically on long timescales. The form of this intrusion is unknown, but it may correspond to the deep crustal sill complex, identified in the recent seismic study⁹ in the vicinity of Oahu.

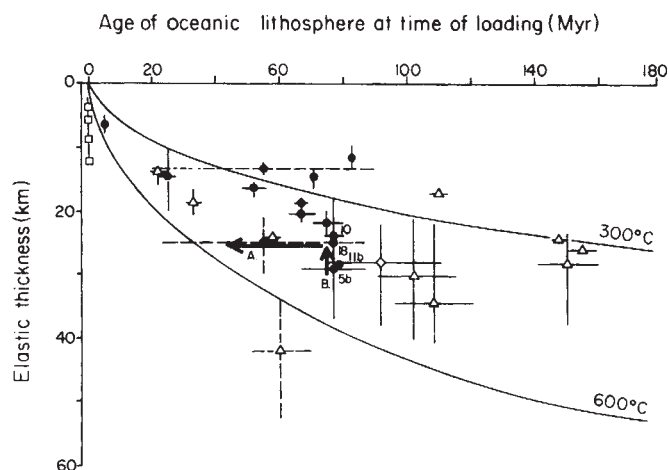


Fig. 4 Plot of the elastic thickness of the lithosphere against age of the lithosphere at the time of loading. ●, Seamount loads; △, trenches; □, mid-oceanic ridge crest topography. Numbered circles are various estimates^{2,9} of elastic thicknesses for the Hawaiian Islands. Arrows represent: A, shift of elastic thickness to a younger apparent age due to regional re-heating, B, shift of elastic thickness to an average lower value due to local thermal perturbation. Solid lines are the 300°C and 600°C oceanic isotherms based on a cooling plate model.

The inferred perturbation of the oceanic geotherm associated with volcano building has important implications for the relationship between elastic thickness and age of oceanic lithosphere at the time of loading¹⁴. According to this relationship, the elastic thickness increases with age, following the depth to the 450°C oceanic isotherm. However, if thermal perturbations of the ocean geotherm occur, then some modifications of this relationship is expected. Figure 4 shows that if the regional geotherm is perturbed in the manner described here, then the plotted position of the elastic thickness at Oahu would have to be shifted from its age at the time of loading (~ 80 Myr) to its equivalent thermal age (44 Myr). In this case, the Oahu value will better fit the depth to the 550°C. If, on the other hand, a local re-heating occurs and elastic thickness varies laterally (Fig. 3), then the observed average elastic thickness will shift towards lower values.

The seismic reflection profile described here is, we believe, the first that was collected in a flexural moat flanking a large volcanic island in the interior of a plate. In order to understand fully the thermo-mechanical setting of large volcanic loads, more data will be required in flexural moats. It will be of particular importance to document the age relationships of the stratigraphical units in the moat and the subsidence history of the island by drilling. These data, together with the development of more refined models, will significantly advance our understanding of the mechanics of flexure.

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T-cell recognition of a chimaeric class II/class I MHC molecule and the role of L3T4

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In addition to expressing clonally distributed antigen-specific and major histocompatibility complex (MHC)-restricted receptors, T cells also express non-clonally distributed surface molecules that are involved in T-cell function. Among the most intriguing of the latter are L3T4 and Lyt 2, which are expressed on individual T lymphocytes in striking, though not absolute, concordance with their restriction by either class II or class I MHC determinants¹⁻⁵, and which are thought to contribute to the overall avidity of T-cell interactions by binding to monomorphic determinants on class II and class I MHC molecules, respectively¹⁻⁹. To examine the ability of T cells to recognize a single class II domain in the absence of the remainder of the Ia molecule, as well as to evaluate the structural basis for the putative interaction of L3T4 with Ia, a recombinant class II/class I murine MHC gene was constructed and introduced into mouse L cells¹⁰. Here we demonstrate that a subset of class II allospecific cytotoxic T lymphocytes (CTL) can specifically recognize and lyse L-cell transfectants expressing an isolated polymorphic $A_{\beta 1}$ domain, and that anti-L3T4 antibody can block such killing, a result inconsistent with the highly conserved membrane-proximal domains of Ia acting as unique target sites for L3T4 binding.

The recombinant class II/class I MHC gene used in the present study (illustrated schematically in Fig. 1) consists of the exons encoding the leader and β_1 domains of the A_{β} class II molecule covalently linked to the exons encoding the C2, transmembrane (TM) and intracytoplasmic (I) domains of the D^d class I molecule. This hybrid gene ($A_{\beta 1}/D_{C2}^d$) encodes a chimaeric MHC molecule that is expressed on the surface of the transfectant L-cell line JT6.4.1 as a membrane-bound glycoprotein of relative molecular mass 30,000–40,000 that is minimally associated with β_2 -microglobulin¹⁰. This membrane protein consists of two external domains, of which the outermost (NH₂-terminal) domain ($A_{\beta 1}$) is a class II MHC gene product while the membrane-proximal domain (D_{C2}^d) is a class I MHC gene product. This molecule can be immunoprecipitated by either monoclonal antibody 10-2.16 (anti-I-A^k) or monoclonal antibody 34-2.12 (anti-H-2D^d). Thus, in contrast to the usual cell-surface expression of a two-chain heterodimer typical of class II MHC antigens, JT6.4.1 L cells are novel in that they express on their cell surface an isolated class II ($A_{\beta 1}$) domain independently of any other class II MHC domains.

To determine first if the isolated $A_{\beta 1}$ domain expressed by JT6.4.1 cells could be recognized by I-A^k allospecific T cells, anti-I^k CTL lines were generated by repetitive *in vitro* stimulation of unprimed B10.T(6R) spleen cells (whose *H-2K*, *I-A*, *I-E* *H-2D* alleles are referred to as *q*, *q*, *-*, *d*) with I^k-disparate B10.AQR (*q*, *k*, *k*, *d*) stimulator cells in the presence of concanavalin A (Con-A)-induced supernatant (ConASN)¹¹. Figure 2a–c shows that lines I and II contained I-A^k subregion-specific CTL as they lysed B10.AQR and B10.A(4R) but not B10.T(6R) lipopolysaccharide (LPS)-induced target cells, and their lysis of B10.A(4R) target cells was specifically inhibited by 10-2.16 anti-I-A^k monoclonal antibody, which did not inhibit the lysis of the same B10.A(4R) target cells by an anti-H-2^k class I allospecific CTL line. In addition to I-A^k-specific CTL, lines I and II were found to also contain I-E^k-specific CTL (data not shown), consistent with their greater lysis of B10.AQR than B10.A(4R) targets (Fig. 2).

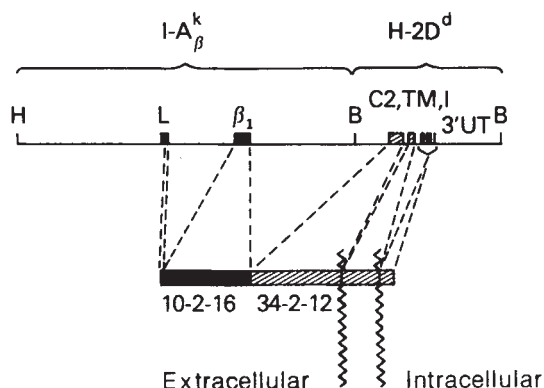


Fig. 1 Schematic diagram of the class II/class I recombinant gene ($A_{\beta 1}/D_{C2}^d$) and its protein product. Details of the construction are given elsewhere¹⁰. The recombinant gene contains the 5'-flanking region, leader exon (L), first intron, β_1 exon, and part of the second intron of A_{β} covalently linked to the 3' part of $H-2D^d$, including part of the third intron, the C2, transmembrane (TM) and intracytoplasmic (I) exons to the 3'-untranslated (3'UT) region. Key restriction endonuclease sites used in the construction are shown: H, *Hind*III; B, *Bam*HI. Boxes represent the indicated exons. The domain structure of the protein product encoded by the corresponding exons is shown. Broken lines connect the gene exons to the corresponding protein domains. Monoclonal antibodies 10-2.16 (ref. 20) and 34-2.12 (ref. 21), which react with determinants encoded by the β_1 domain of I-A^k and the C2 domain of $H-2D^d$ respectively, bind to the corresponding domains of the chimaeric MHC molecule¹⁰.

The ability of anti-I^k lines I and II to recognize and lyse various L-cell transfectants was then assessed (Fig. 2d–f). Both lines effectively lysed D3.11H3 L-cell transfectants expressing conventional I-A^k determinants on their cell surface¹². Control L-cell transfectants prepared using only the herpesvirus thymidine kinase (*tk*) gene (JT1.1) were lysed significantly less well (Fig. 2d, e). The lysis of control L cells was variable and was probably mediated by nonspecific lymphokine-activated killer cells¹³ propagated along with the I^k-specific CTL in our ConASN-supplemented cultures, a complication of most experiments using L-cell transfectants as targets for CTL lines. More interestingly, lines I and II also effectively lysed JT6.4.1 L-cell transfectants expressing the chimaeric $A_{\beta 1}/D_{C2}^d$ MHC molecule. Such lysis was usually first observed after the third cycle of stimulation. Over multiple experiments, lysis of JT6.4.1 cells was less than that of D3.11H3 cells expressing complete I-A^k molecules, even though JT6.4.1 cells had a greater number of 10-2.16-reactive molecules on their membranes (data not shown). It would be expected that lysis of JT6.4.1 L cells by lines I and II is caused by recognition of determinants associated with the $A_{\beta 1}$ domain rather than recognition of D_{C2}^d -related epitopes because: (1) lines I and II were generated by stimulation with an I-region disparity alone and so should not contain class I-specific CTL, and (2) B10.T(6R) responder T cells are themselves H-2D^d and should be genetically tolerant to D_{C2}^d -expressed determinants. This expectation was supported by the fact that the same CTL lines failed to lyse DMT9.9.4 L-cell transfectants which express the same membrane-proximal class I D_{C2}^d domain as JT6.4.1 but which do not express the class II $A_{\beta 1}$ domain¹⁴. Note that all four L-cell targets were equally lysed by class I allospecific anti-H-2^k CTL effectors (Fig. 2f).

These data suggested that at least a subset of I-A^k allospecific CTL could recognize the isolated $A_{\beta 1}$ domain expressed by JT6.4.1 cells, and that they did not require either an $A_{\beta 2}$ domain or an A_{α} polypeptide chain for recognition. To confirm the specificity of lines I and II for the isolated $A_{\beta 1}$ domain, we attempted to block their lysis of JT6.4.1 L-cell targets by using 10-2.16 anti-I-A^k monoclonal antibody, which binds to an epitope on the β_1 domain of the chimaeric MHC molecule^{10,12} (Fig. 3). The ability of monoclonal antibody 10-2.16 to block

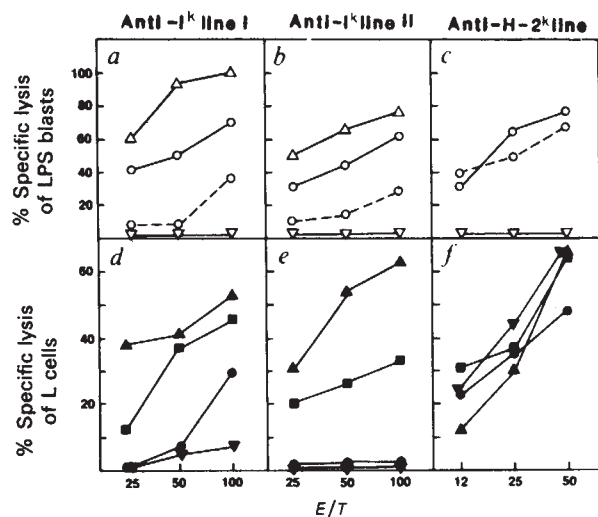


Fig. 2 Lysis of LPS blasts and transfectant L cells by anti- I^k CTL lines. **a-c**, The anti- I^k specificity of lines I and II is demonstrated using LPS-induced blast cells labelled with ^{51}Cr as target cells (open symbols) in a 4-h ^{51}Cr -release assay at the indicated effector to target (E/T) ratios¹¹. Per cent specific lysis was determined according to the formula $100 \times (\text{experimental} - \text{spontaneous release}) / (\text{maximum} - \text{spontaneous release})$. Targets used: ∇ , B10.T(6R) ($q, q, -$, d); Δ , B10.AQR (q, k, k, d); and $\circ$, B10.A(4R) ($k, k, -$, b). The haplotypes are presented as the $H-2K, I-A, I-E$ $H-2D$ alleles respectively. The specificity of these CTL lines for $I-A^k$ -subregion determinants is shown by the ability of monoclonal antibody 10-2.16 (anti- $I-A^k$) to block lysis of B10.A(4R) target cells (dashed lines) and the inability of the same monoclonal antibody to block lysis of B10.AQR target cells by class I allo-specific anti- $H-2K^k$ effectors (**c**). **d-f**, the ability of anti- I^k lines I and II to lyse ^{51}Cr -labelled L cells (closed symbols) was determined in a 6-h ^{51}Cr -release assay. L-cell lines used as targets were: $\bullet$, JT1.1, L-cell line transfected with herpesvirus tk gene alone¹⁰; $\blacktriangle$, D3.11H3, L-cell line transfected with $tk + A_{\beta}^k + A_{\alpha}^k$ genes, expressing $I-A^k$ determinants¹²; $\blacksquare$, JT6.4.1, L-cell line transfected with $tk + A_{\beta}^k / D_{C2}^d$ recombinant class II/class I MHC gene, expressing the chimaeric MHC molecule described in Fig. 1¹⁰; and $\blacktriangledown$, DMT9.9.4, L-cell line transfected with $tk + D_{C2}^d$, expressing the membrane-proximal C2 domain of $H-2D^d$ (ref. 14). The serological phenotype of each L-cell transfectant line was confirmed before assay. Each experimental point represents the mean of triplicate cultures. Standard errors were always $<5\%$ of the mean.

Methods. Anti- I^k lines I and II (**a, b, d, e**) were generated from unprimed B10.T(6R) spleen cells by repetitive stimulations *in vitro* with irradiated (2,000 R) I-region-disparate B10. AQR stimulator cells in the presence of 25% (v/v) Con A-induced spleen cell supernatants (ConASN) as a source of growth factors¹¹. All data shown were derived from T-cell lines after a minimum of four rounds of restimulation. The anti- $H-2K^k$ CTL line (**c, f**) was generated from B10.D2 anti-B10.BR cultures.

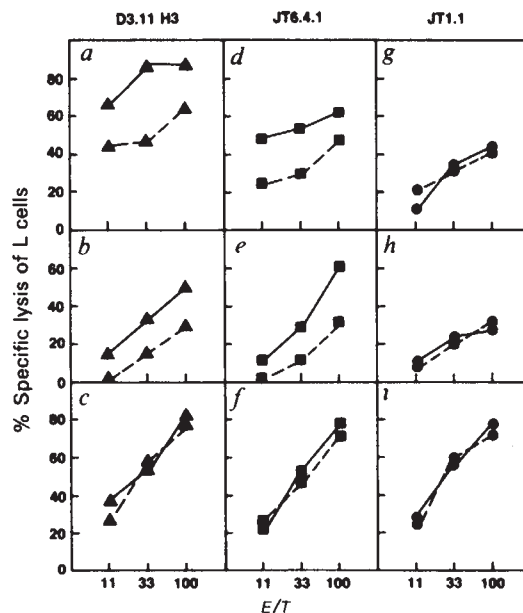


Fig. 3 Lysis of L cells expressing the chimaeric class II/class I MHC molecule (A_{β}^k/D_{C2}^d) by $I-A^k$ allo-specific CTL is blocked by anti- $I-A^k$ monoclonal antibody. L-cell lines were ^{51}Cr -labelled, then incubated with either medium alone (—) or anti- $I-A^k$ monoclonal antibody 10-2.16 (---) at $25 \mu\text{g ml}^{-1}$ final concentration for 30 min at 37°C . At that time, anti- I^k line I (**a, d, g**), anti- I^k line II (**b, e, h**), or anti- $H-2K^k$ CTL effectors generated in B10.AQR anti-B10.A primary cultures (**c, f, i**), were added to the target cells at the indicated E/T ratios for 6 h. The 10-2.16 blocking monoclonal antibody was present during the entire 6-h ^{51}Cr -release assay. Per cent specific lysis was calculated according to the formula in Fig. 2 legend. Lysis of control B10.T(6R) LPS targets was $\leq 2\%$.

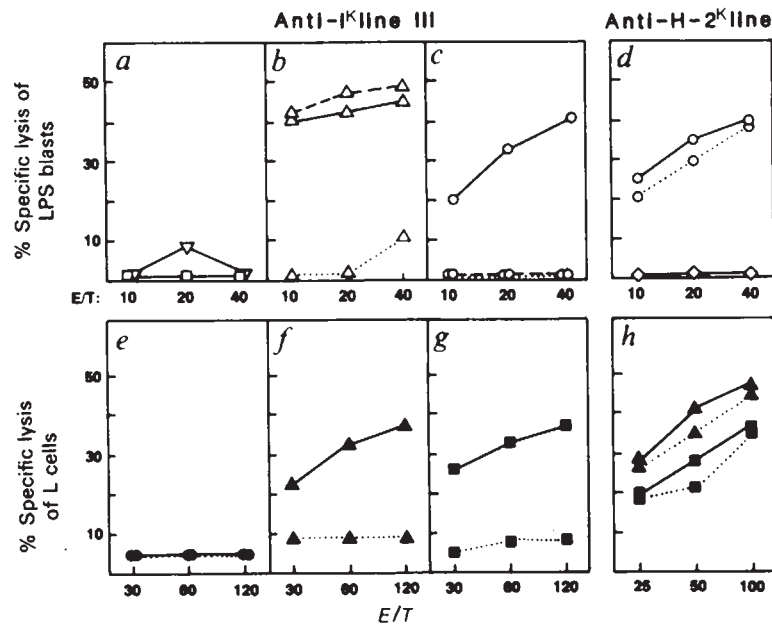
molecule can be recognized by a subset of class II allo-specific T cells.

As the chimaeric MHC molecule expressed by JT6.4.1 L cells lacks both the β_2 and α_2 membrane-proximal domains of Ia, it was especially interesting to examine whether $L3T4^+ I-A^k$ allo-specific CTL could lyse JT6.4.1 L cells, and whether the $L3T4$ molecule has any role in such lysis. Lines I and II were found not to be useful for answering this question as phenotyping of these lines by immunofluorescence revealed that both were predominantly $L3T4^- \text{Lyt } 2^+$, which is a commonly observed phenotype among long-term class II allo-specific CTL lines and clones¹⁵. Consequently, to generate an anti- $I-A^k$ CTL line (line III) that was entirely $L3T4^+ \text{Lyt } 2^-$, B10.T(6R) $\times$ B10.D2 responder spleen cells were treated with anti- $\text{Lyt } 2$ monoclonal antibody plus complement on day 0 before the initiation of culture and again on day 7 after primary sensitization, to deplete the culture of $\text{Lyt } 2^+$ cells. These responder cells were repetitively stimulated at weekly intervals with I^k -disparate irradiated B10.AQR stimulators in the presence of ConASN. At week 4, line III was phenotyped by immunofluorescence and $>99\%$ of the cells were found to be $L3T4^+ \text{Lyt } 2^-$ (data not shown). Simultaneously, the MHC specificity of line III was determined (Fig. 4). Line III lysed I^k -bearing B10.AQR (q, k, k, d) and B10.A(4R) ($k, k, -$, b) LPS targets, but lysed neither B10.T(6R) ($q, q, -$, d) nor B10.D2 (d, d, d, d) LPS targets (Fig. 4a-c). That line III contained $I-A^k$ subregion-specific CTL is shown by the fact that anti- $I-A^k$ monoclonal antibody 10-2.16 specifically inhibited lysis of B10.A(4R) target cells (which express only $I-A^k$ class II molecules) but did not inhibit lysis of B10.AQR target cells (which express both $I-A^k$ and $I-E^k$ class II molecules) (Fig. 4b, c). In addition, consistent with its $L3T4^+ \text{Lyt } 2^-$ phenotype, lysis by line III of B10.AQR and B10.A(4R) LPS targets was inhibited completely by monoclonal antibody anti- $L3T4$, whereas lysis of these targets by a $\text{Lyt } 2^+$ class I anti- $H-2K^k$ CTL line was not (Fig. 4b-d).

Line III was next tested for its ability to lyse L-cell targets. Background lysis of JT1.1 control L cells by line III was always

specifically the lysis of L-cell targets by anti- $I-A^k$ CTL is shown by the fact that the monoclonal antibody reduced the lysis of $I-A^k$ -expressing D3.11H3 L cells by lines I and II to the level of nonspecific background lysis observed on JT1.1 control L cells (Fig. 3a, b, g, h). More importantly, anti- $I-A^k$ monoclonal antibody 10-2.16 also inhibited the lysis of JT6.4.1 L cells expressing the chimaeric MHC molecule to the level of background lysis observed on JT1.1 control L cells (Fig. 3d, e); this inhibition was specific in that monoclonal 10-2.16 antibody did not inhibit the nonspecific background lysis of control JT1.1 L cells by lines I and II (Fig. 3g, h), and did not inhibit the class I-specific lysis of any of the L cells by anti- $H-2K^k$ CTL (Fig. 3c, f, i). Note that, in contrast to the blocking effect of anti- $I-A^k$ monoclonal antibody 10-2.16, lysis of JT6.4.1 L cells by lines I and II was never inhibited by anti- D_{C2}^d monoclonal antibody 34-2.12, which binds to an epitope present on the membrane-proximal class I domain of the chimaeric MHC molecule¹⁰ (data not shown). Thus, these data demonstrate that the outermost domain of the class II/class I chimaeric MHC

Fig. 4 Lysis of L-cell transfectants expressing the chimaeric MHC molecule by L3T4⁺ Lyt 2⁻ anti-I-A^k allospecific CTL. **a-c**, Specificity of line III, using LPS targets (open symbols) from: ▽, B10.T(6R) (*q, q, -*, *d*); □, B10.D2 (*d, d, d, d*); △, B10.AQR (*q, k, k, d*); ○, B10.A(4R) (*k, k, -*, *b*); and ◇, B10 (*b, b, -*, *b*). Lysis of B10.AQR targets (which express both I-A^k and I-E^k class II molecules) and B10.A(4R) targets (which express only I-A^k class II molecules) was carried out either in the absence of blocking antibodies (—) or in the presence of: 25 µg ml⁻¹ 10-2.16 anti-I-A^k monoclonal antibody (---) or 25% culture supernatant of H129.1a anti-L3T4 monoclonal antibody (·····). Anti-I-A^k monoclonal antibody blocked lysis of B10.A(4R) LPS target cells by line III (**c**) but did not block lysis of B10.AQR LPS target cells by line III (**b**), demonstrating that inhibition of lysis by anti-I-A^k monoclonal antibody was specific and that line III contained both I-A^k and a predominance of I-E^k subregion-specific CTL. Anti-L3T4 monoclonal antibody blocked the anti-I-A^k cytotoxic activity of line III (**b, c**) but did not interfere with the lysis of these same LPS targets by a class I anti-H-2^k allospecific line (**d**). **e-h**, Ability of anti-I^k line III and the anti-H-2^k line to lyse L-cell transfectants. CTL effectors in graded numbers were preincubated in microtitre wells with either medium alone (—) or H129.1a anti-L3T4 monoclonal antibody (·····) at 37 °C for 30 min, at which time 1,500 ⁵¹Cr-labelled L-cell targets were added to each well. Per cent specific lysis was determined at the end of the 6-h ⁵¹Cr-release assay. L-cell targets used: ●, JT1.1 (*rk* only) (**e**); ▲, RT4.15HP (*rk + A_{β1}^{k/d} + A_α^k*)¹² (**f, h**); and ■, JT6.4.1 (*rk + A_{β1}^k / D_{C2}^d*) (**g, h**).



Methods: Anti-I^k line III was generated from B10.T(6R) × B10.D2 spleen cells that had been treated with anti-Lyt 2.2 monoclonal antibody (83-12-5 IgM, k) plus complement on days 0 and 7 of culture. These Lyt 2⁻ responders were stimulated weekly with I^k-disparate B10.AQR stimulators in cultures supplemented with ConASN (25% v/v). The anti-H-2^k CTL line was generated from B10 spleen cells repetitively stimulated with B10.BR cells in ConASN-supplemented cultures. The T-cell phenotype of anti-I^k line III as determined by direct immunofluorescence and flow microfluorimetry was L3T4⁺ Lyt 2⁻ as >99% of the cells were positively stained by anti-L3T4 (H129.1a) monoclonal antibody²², and <1% were stained with either anti-Lyt 2.2 monoclonal antibody or with a control anti-Leu 1 monoclonal antibody.

negligible (Fig. 4e), indicating that nonspecific background lysis of L cells by *in vitro*-generated killers is probably mediated by Lyt 2⁺ cells. As would be expected, the anti-I-A^k CTL of line III significantly lysed the RT4.15HP L-cell line¹² which expresses polymorphic I-A^k determinants (Fig. 4f). (The β -chain of RT4.15HP is encoded by an exon-shuffled gene encoding the A _{β 1} external domain and an A _{β 2} membrane-proximal domain.) This lysis of RT4.15HP L cells was significantly inhibited by anti-L3T4 monoclonal antibody. Interestingly, line III also lysed JT6.4.1 L cells expressing the chimaeric MHC molecule (Fig. 4g), and such lysis could be specifically blocked by I-A^k-bearing B10.A(4R) cold target inhibitors (data not shown). Furthermore, contrary to the possibility that L3T4 binds to monomorphic determinants expressed on the membrane-proximal domains of Ia, lysis of JT6.4.1 L cells by line III was also markedly inhibited by anti-L3T4 monoclonal antibody (Fig. 4g). The inhibition of JT6.4.1 L-cell lysis by anti-L3T4 monoclonal antibody was specific as this reagent had no effect on the class I-specific lysis of JT6.4.1 or RT4.15HP L cells by Lyt 2⁺ anti-H-2^k CTL (Fig. 4h). Thus, L3T4⁺ anti-I-A^k CTL recognized the isolated class II A _{β 1} domain expressed by the chimaeric molecule, and their lysis of target cells bearing this domain was blocked by anti-L3T4 monoclonal antibody.

The present data reveal two unexpected findings concerning T-cell allorecognition and cell interaction. First, the allospecific CTL analysed here do not require an appropriate A _{α} glycoprotein to recognize the A _{β 1} domain and, in fact, can recognize the isolated A _{β 1} domain in the absence of any other class II domains, even though these CTL were generated in response to complete Ia determinants expressed by normal spleen stimulator cells. This finding contradicts suggestions of an important role for A _{α} -chain polymorphisms in the activation of antigen-specific class II-restricted T hybridomas (R. Lechler, N. Braunstein, F. Ronchese and R.N.G., manuscript in preparation). Elucidating the basis for this difference may prove especially informative. Second, the present data also demonstrate that the function of the L3T4 molecule in T-cell interactions does not require the presence on target cells of any α 1, α 2 or β 2 Ia domains. Hence, these results effectively exclude the possibility that the L3T4

molecule contributes to the avidity of class II-restricted T-cell interactions by exclusively binding monomorphic MHC determinants present on the membrane-proximal domains of Ia. Instead, if L3T4 is a ligand-binding molecule¹⁻⁹, the present results indicate either that its ligand is present in the highly polymorphic β 1 domain of Ia or that its ligand need not be a class II MHC-encoded gene product. Alternatively, the present results are quite compatible with the possibility that L3T4 is not a ligand-binding molecule at all, and that anti-L3T4 monoclonal antibody either disrupts the cell-surface association of the multiple peptide chains involved in the formation of a functional antigen-receptor complex on the T-cell surface, or in some other way results in the generation of a functional 'off' signal for L3T4⁺ T cells¹⁶⁻¹⁹.

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Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein

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The T11 sheep erythrocyte binding glycoprotein¹⁻⁴ [relative molecular mass (*M_r*) 50,000 (50K)] is expressed throughout human T-lymphocyte ontogeny and appears to play an important physiological role in T-cell activation⁵. Thus, the treatment of T cells with certain monoclonal anti-T11 antibodies results in antigen-independent polyclonal T-cell activation as assessed by proliferation and lymphokine secretion. In addition, the majority of thymocytes that have not yet acquired the T3-Ti antigen/major histocompatibility complex (MHC) receptor can be activated to express interleukin-2 (IL-2) receptors through this T11 structure^{6,7}. We show here that the triggering of cytolytic T (*T_c*) cells via T11 causes an antigen-independent activation of the cytolytic mechanism as evidenced by the induction of nonspecific cytolytic activity. Furthermore, T11⁺T3⁺Ti⁻ natural killer (NK) cell clones can also be induced to lyse NK-cell-resistant targets by treatment with anti-T11 monoclonal antibodies directed at defined T11 epitopes. These results indicate that T11 triggering can activate cytotoxic lymphocytes to express their functional programmes in the absence of specific antigen recognition via the T3-Ti complex and provide further evidence for the notion that certain NK cells and T lymphocytes are related.

We have previously identified three distinct epitopes on the 50K T11 molecule using monoclonal antibodies⁵. While the T11₁ and T11₂ epitopes are expressed on resting as well as activated T cells, anti-T11₃ antibodies recognize a spatially distinct epitope that is preferentially expressed on mitogen- or antigen-activated T lymphocytes and thymocytes. Interestingly, the T11₃ epitope can be induced on resting T cells within 30 min at 0°C by treatment with anti-T11₂, thus implying that it is conformational. These studies have also shown that the combination of anti-T11₂ and anti-T11₃ (and to a lesser extent certain anti-T11₁ and anti-T11₃ antibodies) can induce IL-1-independent polyclonal T-cell activation as measured by proliferation and lymphokine secretion.

To determine whether T11 triggering could activate *T_c*-cell effector function, we examined the effect of anti-T11 monoclonal antibodies on the lysis of target cells by alloreactive *T_c*-cell clones (Fig. 1). QQ is a representative T8⁺ *T_c*-cell clone specific for the HLA-B7 molecule expressed by Laz 156, an allogeneic Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell line. As shown in Fig. 1, in the absence of anti-T11 antibodies, this clone lyses Laz 156 but not Laz 509, an EBV-transformed B-cell line derived from a donor possessing a different HLA genotype. Anti-T11₁ strongly inhibits the specific lysis of Laz 156. Similar inhibitory effects have been observed with other anti-T11 antibodies that block rosetting^{8,9}. However, saturating concentrations of anti-T11₂ or anti-T11₃ give only minimal inhibition of specific cytotoxicity. More importantly, anti-T11₃ in combination with either anti-T11₁ or anti-T11₂ induces clone QQ to lyse the inappropriate lymphoblastoid target Laz 509. Monoclonal antibodies to other cell-surface markers expressed by QQ (anti-Ti) or not expressed by QQ (anti-T4 and anti-T6) separately (see Fig. 1) or together (not shown) have no such effect. Thus, anti-T11-induced cytotoxicity results from the synergistic effects of particular combinations of anti-T11 monoclonal antibodies (anti-T11₁ plus anti-T11₃ or anti-T11₂ plus anti-T11₃). This result suggests that cross-linking of T11 may be important in *T_c*-cell

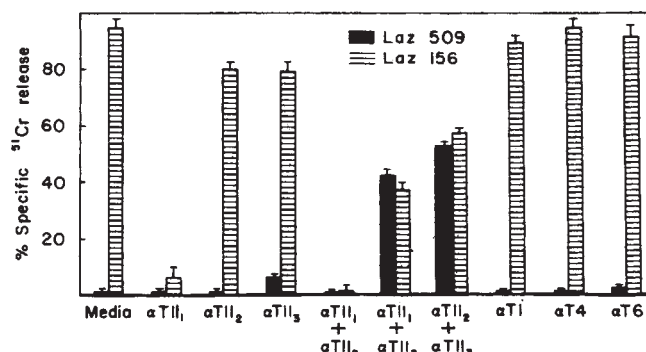


Fig. 1 Lysis of ⁵¹Cr-labelled B-lymphoblastoid target cell lines Laz 509 and Laz 156 by the alloreactive *T_c*-cell clone QQ in the presence and absence of anti-T11 monoclonal antibodies (anti-T11). Clone QQ is a T3⁺T4⁺T8⁺T11⁺ *T_c*-cell clone specific for the HLA-B7 molecule on the allogeneic EBV-transformed B-cell line Laz 156 (HLA genotype: A2, A3, B7, B40, Dr2,4). *T_c*-cell clones specific for Laz 156 were isolated as previously described³⁵.

Methods. Peripheral blood mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation were stimulated with irradiated (5,000 R) Laz 156 and cloned on day 5 in soft agar. Clones were expanded in liquid culture by restimulation with irradiated Laz 156 and IL-2-containing supernatants. The surface phenotype was determined by indirect immunofluorescence on an Epics V cell sorter as described previously²⁸. Laz 509 is an EBV-transformed B-cell line from an HLA-disparate donor (HLA genotype: A2, A25, B13, Bw38, Cw6, Dr7). The monoclonal antibodies anti-T11_{1C} (IgG2b), anti-T11₂ (IgG2a) and anti-T11₃ (IgG3) were produced and characterized as described elsewhere⁵ and were used in ascites form at a 1:100 final dilution. In other experiments, dose-dependent blocking and activating effects have been observed at dilutions of ascites between 1:100 and 1:1,000 and at concentrations of purified antibody of 1–100 µg ml⁻¹. Control antibodies to other T-cell surface structures such as Ti and irrelevant control antibodies are consistently negative for these effects. Isotype-matched irrelevant control antibodies anti-T4 (IgG2a) and FLOPC 21 (IgG3) alone or in combination are consistently negative in these experiments at dilutions of ascites as high as 1:100. Lysis was measured using a standard ⁵¹Cr-release assay in which *T_c* cells and labelled targets were mixed at a 3:1 ratio in the presence of the indicated concentrations of antibody in a final volume of 0.2 ml. After centrifugation (1,200 g, 10 min), assay plates were incubated for 4 h at 37°C before re-centrifugation and removal of aliquots of supernatant for assay of γ radioactivity.

activation, although other factors appear to be involved because the combination of anti-T11₁ and anti-T11₂ does not induce activation and because previous studies have shown that anti-T11₂ or anti-T11₃ alone cannot induce T-cell proliferation when coupled to Sepharose⁵.

The induction of antigen-independent cytotoxicity by anti-T11 antibodies clearly results from the action of the antibodies on the *T_c*-cell clone because (1) these targets do not express T11 and (2) the anti-T11 antibodies do not induce lysis of target cells in the absence of *T_c* cells. Furthermore, the effect can also be observed if the *T_c* cells are pretreated with the anti-T11 antibodies (data not shown).

In addition to QQ, two other *T_c*-cell clones, HH and J, were induced to express nonspecific cytolytic activity by treatment with anti-T11₂ plus anti-T11₃ (Fig. 2). In contrast, the two alloreactive non-cytotoxic inducer clones, AA7 and AA10, were not triggered to kill by treatment with anti-T11₂ and anti-T11₃ antibodies. Thus, it appears that T lymphocytes that have acquired cytolytic function during differentiation can be induced to lyse a variety of target cells in an apparently nonspecific fashion by treatment with appropriate combinations of anti-T11 antibodies. On the other hand, cytotoxicity is not induced in clones whose active genetic programme lacks the cytotoxic machinery. A similar finding was noted in earlier lectin approximation studies¹⁰. Because mitogenic lectins bind to T11 and the T3-Ti complex^{11,12}, this similarity is perhaps not surprising.

To determine whether anti-T11-induced cytotoxicity occurs

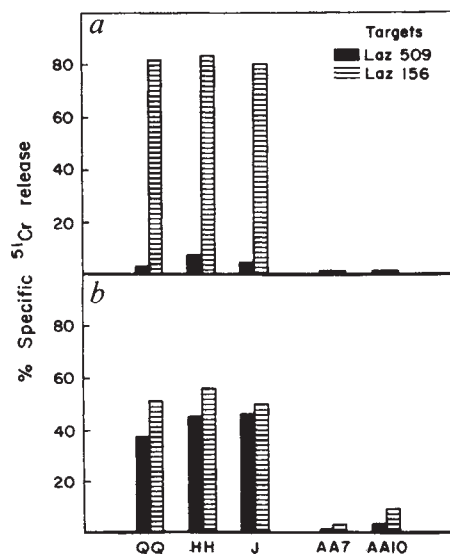


Fig. 2 Effect of anti-T11 antibodies on cytolytic effector function of various T-cell clones. HH and J are T8⁺ alloreactive T_c-cell clones (phenotype T3⁺T4⁺T8⁺T11⁺) generated by stimulation with Laz 156. AA7 and AA10 are alloreactive non-cytolytic T-cell clones (phenotype T3⁺T4⁺T8⁺T11⁺) generated by stimulation with Laz 509. The effect of these clones on ⁵¹Cr release by Laz 509 and Laz 156 was examined in the absence of anti-T11 antibodies (a) and in the presence of anti-T11₂ and anti-T11₃ (b). These clones were isolated and characterized as described above for QQ. Antibodies were used in ascites form at a 1:100 final dilution. Lysis was measured in a standard ⁵¹Cr-release assay at an effector/target ratio of 10:1.

through effector mechanisms analogous to those involved in specific cytotoxicity, we compared antigen-specific and anti-T11-induced cytotoxicity with respect to parameters affecting cytolytic activity, including calcium requirements, temperature sensitivity, cell-contact dependence and susceptibility to inhibition by anti-T8 antibodies. The results are summarized in Table 1. Calcium is known to be required in the lethal hit stage of antigen-specific cytotoxicity¹³⁻¹⁵. We found that it is also required for anti-T11-induced cytotoxicity because lysis is completely inhibited by EGTA. In addition, both antigen-specific and anti-T11-induced cytotoxicity are inhibited by low temperature. However, anti-T11-induced cytotoxicity appears to be more temperature sensitive because lysis of Laz 509 and Laz 156 in the presence of anti-T11 antibodies is completely inhibited at 25 °C whereas specific killing of Laz 156 by QQ is only reduced by ~50%. Furthermore, no stable cytolytic factor is detected in the supernatants of T_c-cell clones triggered with anti-T11₂ plus anti-T11₃ in this assay system. Consequently, it is likely that either cell contact or close effector-target cell proximity is required for anti-T11-induced cytotoxicity. This would not be unexpected if T11 triggering results in the exocytosis of granules containing a presumptive T-cell lysis factor that is analogous to bacterial toxins or the terminal lytic complement complex and hence unstable in solution^{16,17}. Evidence for such lytic granules has been obtained for both T_c and NK cell clones^{18,19}.

These mechanistic similarities between antigen-specific and anti-T11-induced killing suggest that T_c cells damage target cells in the same way whether activated through the T3-antigen receptor pathway or via the T11 molecule. Although T11 triggering induces lymphotoxin (LT) secretion by T-cell clones, it is unlikely that anti-T11-induced cytotoxicity is mediated by LT alone because the anti-T11-induced lytic effect can be measured in a 4-h assay using a variety of target cells, including those which are resistant to the effects of LT. In contrast, the detection of LT-mediated cytotoxicity generally requires sensitive target cell lines, longer assays and prior target cell conditioning^{20,21}.

Antigen-specific and anti-T11-induced cytotoxicity differ with respect to susceptibility to inhibition by antibodies to T8 (Table

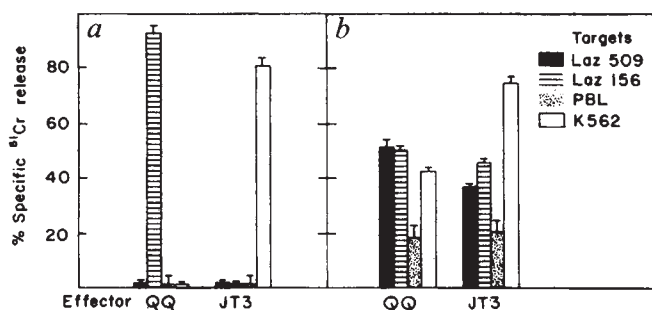


Fig. 3 Lysis of various target cells by T_c-cell clone QQ and by NK cell clone JT3 in the absence (a) and presence (b) of anti-T11₂ and anti-T11₃. JT3 is a T3⁺T11⁺ NK cell clone that has been described in detail elsewhere²³. Targets include Laz 509, Laz 156, allogeneic peripheral blood lymphocytes isolated by Ficoll-Hypaque density gradient centrifugation, and K562, an NK-cell-sensitive line established from a patient with chronic myelogenous leukaemia. Antibodies were used in ascites form at a 1:100 final dilution. Lysis was measured in a standard 4-h ⁵¹Cr release assay at an effector/target ratio of 10:1.

1). Thus, whereas antigen-specific cytotoxicity by clone QQ is completely inhibited by anti-T8, anti-T11-induced killing by the same clone is virtually unaffected. In this regard, previous studies have shown that anti-T8 inhibits antigen-specific cytotoxicity at the initial conjugate formation step²². Therefore, the failure of anti-T8 to inhibit anti-T11-induced cytotoxicity suggests that the adhesion-promoting function of the T8 molecule may be important for facilitating antigen receptor-target cell interactions but not for the delivery of lethal hits once the cytolytic effector mechanism has been activated. As is the case with anti-T8, a monoclonal antibody (W6/32) to class I MHC gene products inhibited antigen-specific but not anti-T11-induced cytotoxicity (data not shown).

The vast majority of human NK cells, like early thymocytes, lack T_i α-chain messenger RNA, and consequently express no surface T3-Ti complex. Nevertheless, the 50K T11 glycoprotein is found on the surface of most NK cells. These results suggest that certain NK cells may be related to T-lineage precursors²³⁻²⁶, which made it important to examine the effects of anti-T11 antibodies on T11⁺T3⁺ NK cell clones.

As shown in Fig. 3, in the absence of monoclonal antibodies, the previously described²³ representative T11⁺T3⁺ NK clone JT3 efficiently kills the NK-sensitive target K562 but not peripheral blood lymphocytes or either of two B-lymphoblastoid target

Table 1 Comparison of antigen-specific and T11-induced cytotoxicity

Effector	Conditions	Anti-T11 ₂ + anti-T11 ₃	% Specific ⁵¹ Cr release	
			Laz 509	Laz 156
QQ	Control	—	6	81
QQ	Control	+	47	68
QQ	5 mM EGTA	—	0	1
QQ	5 mM EGTA	+	2	7
QQ	25 °C	—	2	42
QQ	25 °C	+	3	5
None	Supernatant*	—	1	0
QQ	Anti-T8	—	6	9
QQ	Anti-T8	+	50	54

The lysis of Laz 509 and Laz 156 by T_c-cell clone QQ was measured in the absence and presence of anti-T11 antibodies in the indicated conditions. Anti-T11 antibodies were used in ascites form at a 1:100 final dilution. In one set of wells, effector cells were omitted and replaced with cell-free supernatants of clone QQ triggered with anti-T11₂ and anti-T11₃ for 4 h. The supernatants were used at a 50% final concentration. The monoclonal antibody to T8 has been described previously¹⁰.

* Supernatant of clone QQ triggered with anti-T11₂ and anti-T11₃ at final dilutions of 1:100 each for 4 h.

lines tested. This specificity pattern is clearly distinct from that of the Laz 156-specific T_c -cell clone QQ. However, after incubation with anti-T11₂ and anti-T11₃ antibodies, both JT3 and QQ kill all of the target cells to varying degrees. Similar data were obtained for the independently derived NK cell clone JT_{B18} (ref. 23). This ability of anti-T11 antibodies to induce killing activity from cells that lack a T3-Ti antigen/MHC receptor complex clearly implies that the T11 molecule represents an alternative pathway of cell activation as documented for inducer cells⁵. More importantly, these findings suggest that the T11 molecule may be a critical structure for inducing cytolytic function in NK cells as well as T_c cells. The question of whether the putative NK cell receptor for specific target antigens is associated with or is unrelated to the T11 structure will require further investigation.

A recent report suggests that certain monoclonal antibodies to the 20–25K T3 molecule can also induce nonspecific cytolytic activity²⁷. Since T3 forms part of the T-cell antigen receptor complex^{28,29}, these antibodies are probably mimicking the physiological effects of antigen. The present study identifies T11 as a second T-cell surface structure through which the cytolytic effector mechanism can be activated. The ability of anti-T11₁ antibodies to inhibit antigen-specific cytotoxicity suggests that the T11 molecule may also be essential for the activation of cytolytic effector function through the T3-Ti complex. This notion is supported by the observation that the combination of anti-T11₂ plus anti-T11₃ induces nonspecific cytotoxicity and yet reduces the level of specific killing to that of nonspecific killing (Fig. 1). These findings suggest that the T3-Ti and T11 pathways work in series rather than in parallel in mature T lymphocytes. Further support for this notion comes from the observation that the T3-Ti pathway regulates activation through T11⁵.

The precise function of the T11 molecule in the physiology of lymphocyte activation remains uncertain. Nevertheless, it is clear that monoclonal antibodies to T11 and to the T3-Ti complex induce rapid transmembrane calcium flux resulting in increased intracellular free calcium concentrations^{30–34}. This alteration appears to be directly or indirectly necessary for IL-2 gene and IL-2 receptor gene activation and for the induction of the cytolytic programme. In fact, the calcium ionophore A23187 induces nonspecific lytic activity of T_c -cell clones similar to that observed with anti-T11₂ plus anti-T11₃ (data not shown). Since the binding of anti-T11 antibody to T11⁺T3⁺ thymocytes induces calcium flux (Alcover *et al.*, submitted), it is obvious that the location of a putative calcium channel is not restricted to the T3 molecules themselves (if, in fact, one exists there). Thus, a membrane calcium channel could exist either within the T11 structure or elsewhere. Alternatively, T11 might function either to affect levels of inositol phosphates, cyclic nucleotides, GTP binding proteins or other ions including sodium, potassium or protons that could alter membrane potential. Primary structural analysis of the T11 molecule and liposome reconstitution studies will be useful in elucidating some of these possibilities.

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Variability and repertoire size of T-cell receptor V_α gene segments

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The immune system of higher organisms is composed largely of two distinct cell types, B lymphocytes and T lymphocytes, each of which is independently capable of recognizing an enormous number of distinct entities through their antigen receptors; surface immunoglobulin in the case of the former, and the T-cell receptor (TCR) in the case of the latter. In both cell types, the genes encoding the antigen receptors consist of multiple gene segments which recombine during maturation to produce many possible peptides^{1–9}. One striking difference between B- and T-cell recognition that has not yet been resolved by the structural data is the fact that T cells generally require a major histocompatibility determinant together with an antigen^{10,11} whereas, in most cases, antibodies recognize antigen alone. Recently, we and others have found that a series of TCR V_β gene sequences^{12,13} show conservation of many of the same residues that are conserved between heavy- and light-chain immunoglobulin V regions, and these V_β sequences are predicted to have an immunoglobulin-like secondary structure^{12,13}. To extend these studies, we have isolated and sequenced eight additional α -chain complementary cDNA clones and compared them with published^{14–16} sequences. Analyses of these sequences, reported here, indicate that V_α regions have many of the characteristics of V_β gene segments but differ ... that they almost always occur as cross-hybridizing gene families. We conclude that there may be very different selective pressures operating on V_α and V_β sequences and that the V_α repertoire may be considerably larger than that of V_β .

Eight cDNA libraries derived from functional T-cell lines or hybrids were screened by standard procedures using α -chain constant-region (C_α) and β -chain constant-region (C_β)-specific probes. One α -chain cDNA clone from each library was sequenced by the procedure of Maxam and Gilbert¹⁷ and the sequences

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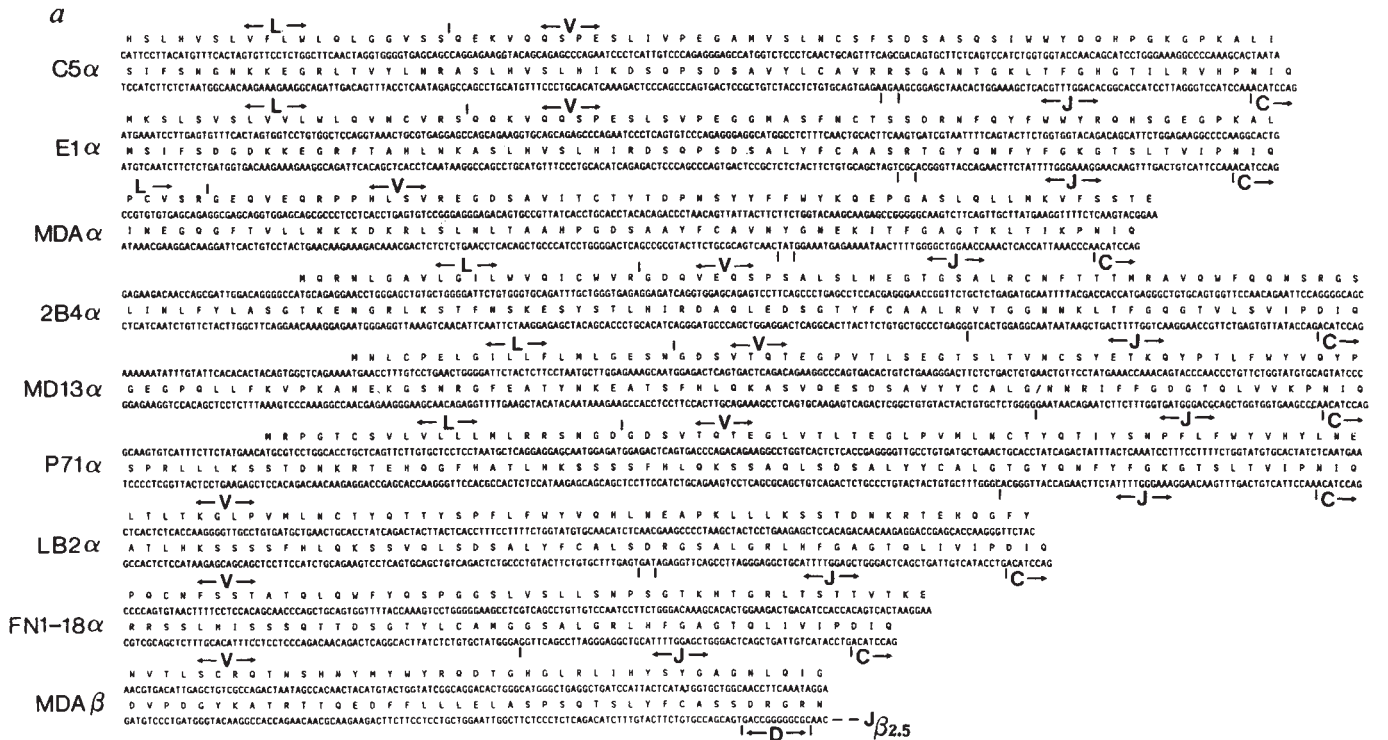
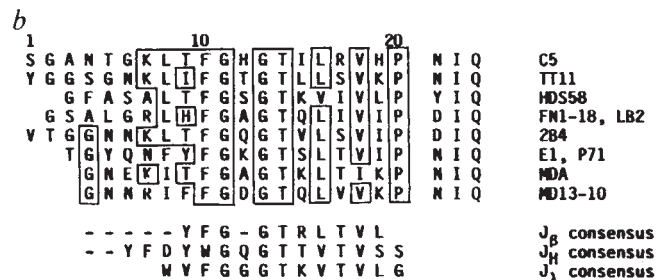


Fig. 1 a, Sequences of variable regions from functional T-cell cDNA libraries. The C5, E1 and LB2 libraries have been reported previously¹². These, as well as the MDA, MD13-10 (abbreviated to MD13) and P71 libraries were all constructed by the method of Okayama and Berg⁴³. The 2B4 library was made using the λgt10 vector⁴⁴ as reported previously², and the FN1-18 library was made in the same fashion. Screening with C_α and C_β radiolabelled probes was by standard procedures. The first three amino acids of the constant region are shown at the end of each α-chain sequence. The leader junction is approximate and has been set at amino acids 19-21, based on V_α homologies to immunoglobulins. The V_α-J_α junction is also approximate and appears to vary from three to four codons 3' to the conserved cysteine at position 92, based on germline V_α sequences (ref. 9 and R. Wallich, unpublished observation). T-cell specificities: of the T-helper cells, C5 responds to the dinitrophenol-ovalbumin (DNP-OVA) antigen and is restricted by I-A^b (ref. 18), E1 responds to trinitrophenol (TNP) on a variety of carriers and is restricted to I-A^d (ref. 18), LB2 and MD13-10 are both reactive with chicken red blood cells (the former restricted by I-A^b and the latter by I-A^d; ref. 19), 2B4 responds to pigeon cytochrome c and is restricted by I-E^k (ref. 20), FN1-18 is specific for keyhole limpet haemocyanin (KLH) and is restricted by I-E^k and a number of other Ia molecules²¹. The two cytotoxic T cells, MDA and P71, are both alloreactive for D^b (ref. 22). Strain origins: C5, LB2 and FN1-18 are of C57BL/6 origin, E1, MDA, P71 and MD13-10 are BALB/c and 2B4 is from B10.A. **b**, Summary of the eight distinct J_α sequences, and comparison with their immunoglobulin equivalents. The first three amino acids of the α-chain constant region are also shown separated from the J_α sequences (see text).



are presented in Fig. 1a; a β-chain cDNA clone from the MDA hybridoma was also sequenced (Fig. 1a). β-Chain sequences for many of the other lines are given in the literature¹². The antigen/major histocompatibility complex (MHC) specificities of the cell lines and their strains of origin are summarized in Fig. 1 legend. Six of these (C5, E1, 2B4, MDA, LB2 and FN1-18)¹⁸⁻²¹ are of the T helper phenotype in that they release interleukin-2 in response to antigen and are Ia (class II)-restricted. Two (MDA, P71)²² are cytotoxic T-cell hybridomas alloreactive for D end molecules of the b haplotype. The V_β-chain sequence of MDA is the same as the TB23 V_β reported by Barth *et al.*¹³. All the α-chain cDNAs used the same constant region, consistent with the finding that there is a single α-chain constant region in the genome⁹. One of the α-chains, MD13-10, appears to represent a frameshift at the V-J junction, the approximate boundary region marked by a solidus in Fig. 1a. This is a relatively common occurrence in immunoglobulins²³, reflecting the imprecision of the joining process²⁴ and examples of this have recently been seen in β-chain genes (ref. 25 and N. Lee *et al.*, in preparation), an indication of the similarity of this mechanism between T-cell receptor genes and those of immunoglobulin. These sequences also indicate the existence of six new J_α gene segments (Fig. 1b); together with the two

mouse sequences in the literature^{19,20}, this makes a total of eight distinct J_αs out of 10 cDNA sequences. This indicates that the J_α repertoire, like that of J_β (ref. 4), is very large. Homologies between these distinct J regions and consensus sequences for J_β, J_H and J_λ are also shown in Fig. 1b. The 5' and 3' borders of the J_α segments are based on the genomic sequence of the TT11 and 2B4 J_α segments (T. Lindsten and R. Wallich, unpublished observations) as well as that of 2C2 (ref. 9). As noted previously¹⁴⁻¹⁶, these J_α sequences have many of the conserved features of immunoglobulin J regions as well as those of the β-chain. Another interesting feature of the J-region comparisons is that both J_α and J_β are quite large; for the former, 17-20 amino acids, and the latter, 15-17 amino acids. This differs from the case of immunoglobulins where J_H comprises 13-19 amino acids and J_λ and J_κ are uniformly 13 amino acids long²⁶. Thus, the size constraints on these segments of the T-cell receptor seem different from those of immunoglobulin and may indicate a slightly different type of structure in that portion of the molecule. In this same context, the J_β sequences are shifted one amino acid N-terminal compared with immunoglobulin J regions (as are the T-cell receptor-like γ-chain genes²⁷) but the J_α sequences are not, again perhaps indicating different structural constraints. Figure 1b also shows the rather variable first

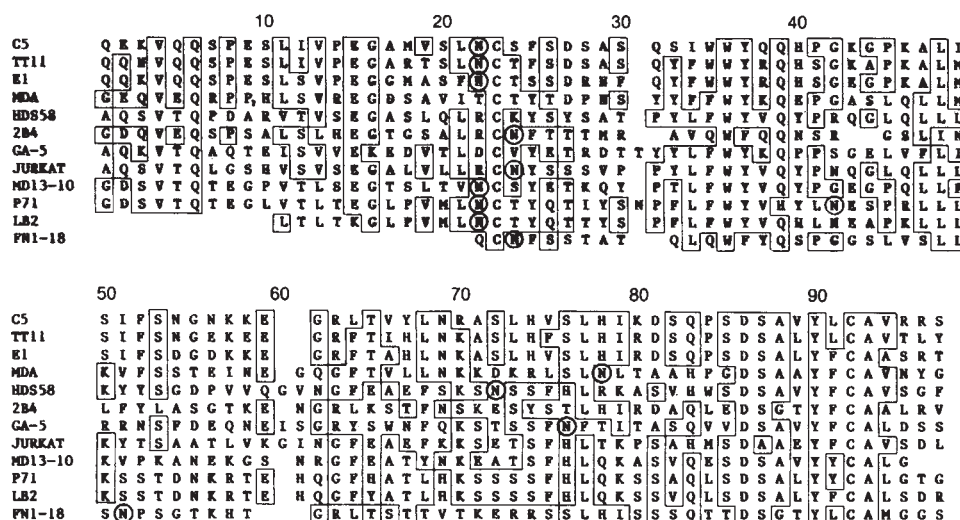


Fig. 2 V_{α} homologies and glycosylation sites. Five or more matches are boxed, except in the first position where four G residues are boxed. In instances where two different residues occur five times, only one is boxed. Possible N -linked glycosylation sites are circled. Numbering system follows that of the largest V_{α} sequence (GA-5). The TT11 V_{α} sequence is from ref. 14 and derives from the BW5147 lymphoma line. The HDS58 V_{α} sequence is from ref. 15. GA-5 is from ref. 16; Jurkat α from refs 29, 30. The remainder are from the present study.

amino acid of the constant region; this variability stems from the fact that the first nucleotide of this codon is derived from the J_{α} segment (ref. 9 and C. Goodnow, unpublished observations).

The T cells which share J_{α} sequences (FN1-18 and LB2, E1 and P71) have neither antigen specificity nor MHC-restricting elements in common (see legend to Fig. 1a), nor is there any obvious correlation between the V_{α} and V_{β} usage. This seems to be another indication of the diversity of the T-cell response. In cases of a very narrowly defined specificity, with a small antigen and the same restricting element, such as pigeon cytochrome c , the same V and J regions of the β -chain are used repeatedly²⁸.

The V_{α} amino-acid sequences, shown in Fig. 1a, were combined with the two mouse^{14,15} and two human sequences^{16,29,30} in the literature and aligned as shown in Fig. 2: identities of five or more residues are boxed and possible N -glycosylation sites are circled. For simplicity, the numbering system follows that of the largest V region. It is interesting to note that a highly conserved N -glycosylation site occurs around the first cysteine at either residue 22 or 24. The fact that such a site occurs in 9 out of 12 cases suggests that N -glycosylation might occur at this position and in fact this is near the point at which N -glycosylation occurs in human V_{κ} sequences³¹. No such conserved site appears in the V_{β} sequences¹².

The V_{α} sequences have 14 invariant or nearly invariant (11/12) residues, all of which are highly conserved in immunoglobulin V regions²⁶, especially light-chain sequences. Particularly interesting is the recent work of Novotny and Haber which indicates that the tyrosines at amino-acid positions 36 and 87 of the immunoglobulin light-chain variable region (V_L), the glutamine at position 38, and the phenylalanine at 98 (in the J region) seem to be very important in the formation of the V_L/V_H domain³². This conservation is another indication that the presumptive binding portion of the T-cell receptor, the V_{α}/V_{β} domain structure, will be very similar to that of immunoglobulin. That it will not be identical is also indicated in the same study, which found that the invariant tryptophan at position 47 of the heavy chain is also important in the V_L/V_H interactions, yet there is no conserved aromatic amino acid at the equivalent position in either the TCR α - or β -chains (Fig. 2 and refs 12, 13). Therefore, the T-cell receptor may, in some respects, resemble a light-chain dimer³³ rather than a V_H/V_L dimer. Such dimers have a characteristically more open structure than V_H/V_L dimers³³. Also consistent with this view is the fact that the distances between the invariant cysteine residues (which presumably form the characteristic intrachain disulphide bond) in V_{α} (63–67 amino acids) (Fig. 2) and V_{β} (67–69 amino acids) fall more within the light-chain range (64–69 amino acids)²⁶ than that of heavy-chain V regions (69–75 amino acids)²⁶.

Pairwise comparisons of the sequences shown in Fig. 2 reveal

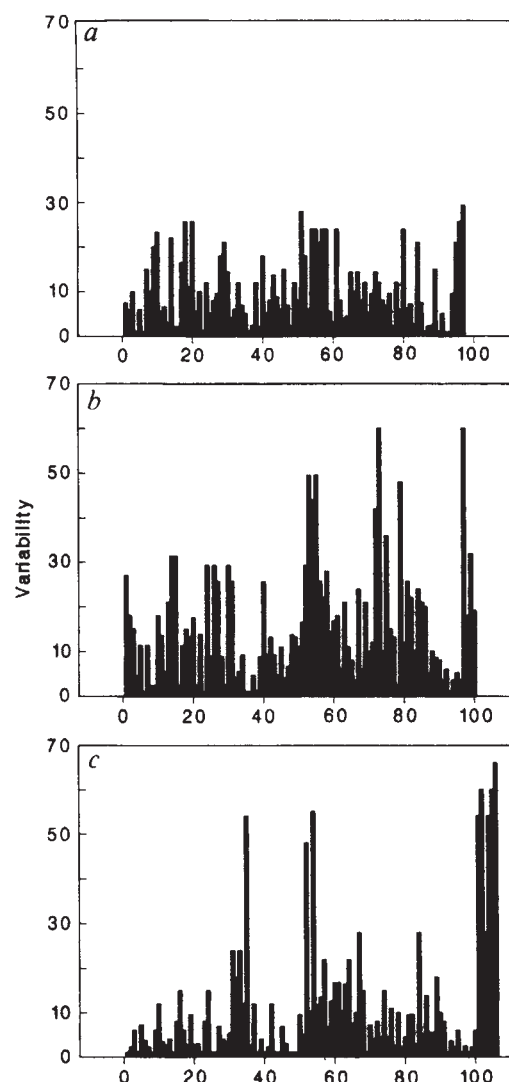


Fig. 3 Variability plots of 12 V_{α} , 12 V_{β} and 12 N-terminally blocked human V_H sequences²⁶, which are thought to be more random than mouse sequences¹³. The V_{β} sequences derive from the following: 86T1 (ref. 45), 2B4.71 (ref. 2), HDS11 (ref. 38), E1 (ref. 12), LB2 (ref. 12), FN1-18 (N.R.J.G., in preparation), BW51.4 (N. Lee, personal communication), C5 (ref. 12), YT35 (ref. 46), HPB-ALL²⁹, HPB-b2 (ref. 47). V-D-J and V-J junctions are retained to allow a reference point. V_{β} s and V_H s are numbered in the same manner as the V_{α} s (see Fig. 3 legend) for ease of comparison.

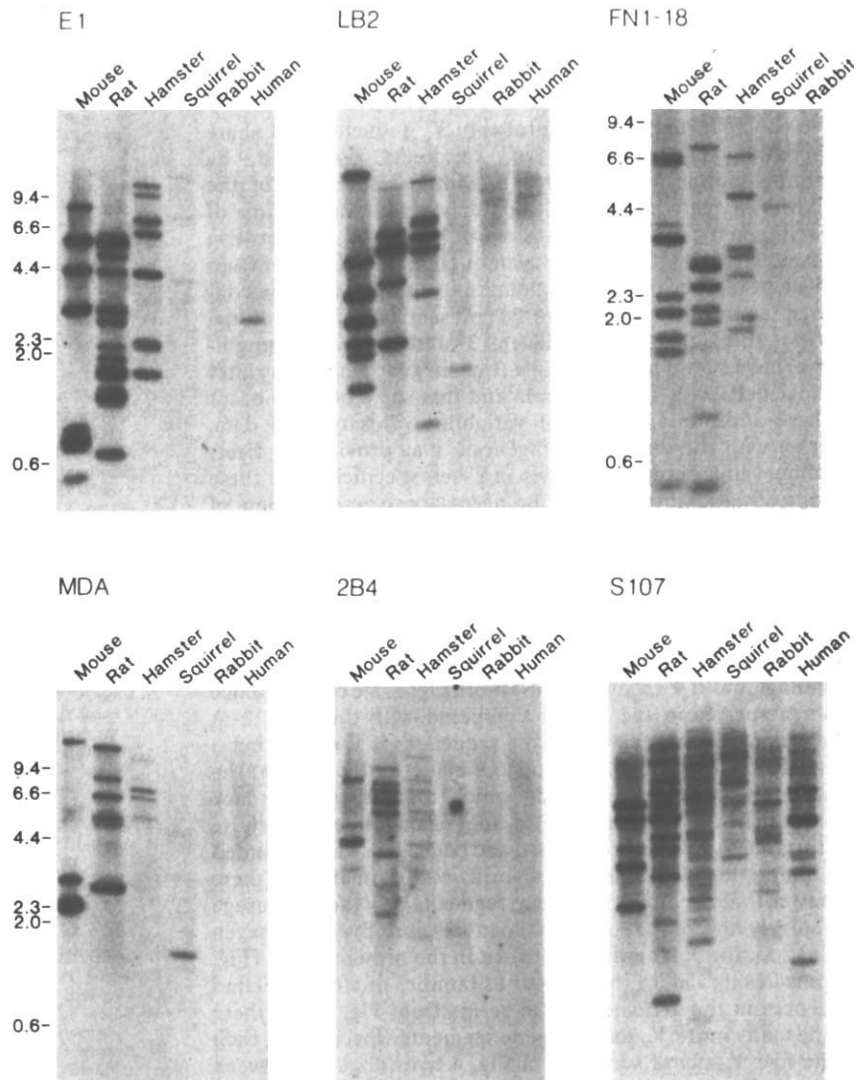


Fig. 4 Cross-hybridizing V_{α} sequences in mouse and other species. *Hind*III digests of murine (C57BL/6), rat, hamster (CHO cells), ground squirrel, rabbit and human DNAs were electrophoresed through 0.9% agarose and blotted onto nitrocellulose by standard procedures. Inserts were isolated from V region-specific subclones of five of the seven distinct families of V_{α} cDNA clones and nick-translated and hybridized for 12–16 h at 55 °C in $6\times$ SSC, washed with $2\times$ SSC at the same temperature, followed by a final wash with $0.2\times$ SSC for 30 min (again at 55 °C). At this stringency, we are able to visualize V -region sequences which are as little as 70% homologous (R. Mage, personal communication). Size markers are indicated in kilobases. Divergence times from mouse (Myr)¹²: rat, 15; hamster, 25; ground squirrel, 60; rabbit, 70; human, 90.

an average homology of 36% (standard deviation (s.d.) = 13%); this compares with 30% (s.d. 11%) for a similar comparison of 12 V_{β} sequences and indicates that both V_{α} s and V_{β} s are significantly more divergent from each other than similar numbers of randomly chosen V_H s (48–51%, s.d. 11–13%). This was also observed for V_{β} s using a homology plot¹². Although this difference in divergence may be functionally significant (see ref. 12 and below), the predicted secondary structure for V_{β} s^{12,13} and V_{α} s (J. Novotny, personal communication) is very similar to that of immunoglobulins, again indicating a basic, although not necessarily exact, conservation of structure. These comparisons of V_{α} sequences also indicate that C5, TT11 and E1 are members of a cross-reactive family of sequences with amino-acid homologies of 80–90% and nucleic acid homologies of ~90%. P71 and LB2 also constitute a distinct family by this criterion.

Wu and Kabat³⁴ were the first to use variability analysis of immunoglobulin V -region sequences in an effort to predict which regions might be involved in binding to antigen. Subsequent structural analysis^{35,36} showed that the three principal peaks of variability that are seen in both heavy- and light-chain sequences²⁶ comprise the major portion of the immunoglobulin binding site. Although variability analysis is no substitute for the appropriate structural data, it may provide testable hypotheses concerning the nature of T-cell receptor–ligand interactions. Variability plots for the 12 V_{α} sequences discussed here are shown in Fig. 3, together with a plot of 12 V_{β} sequences and 12 human V_H sequences (see Fig. 3 legend). It is important to keep the number of V -region sequences constant as these analyses are very sensitive to the number of sequences compared,

especially in the lower ranges. In these analyses, we have used both mouse and human sequences as the number available is still so small that each new sequence adds to the reliability of the plot, outweighing the slight increase in noise that may result. V -region sequences are also continued into the D and J elements because these make up the third hypervariable region of immunoglobulin and provide an additional reference point.

The data indicate the following: V_{α} sequences are not as variable at any major peak as either V_H or V_{β} , but recognizable peaks in the area of the second and third hypervariable regions of immunoglobulin are apparent. However, the first hypervariable region seems significantly less variable than in immunoglobulin and the region in residues 1–23 seems more variable, and is similar in its overall pattern to the same region in V_{β} . In contrast, this portion of the immunoglobulin is highly conserved. These data also tend to confirm the view of Patten *et al.*¹² that V_{β} sequences have additional regions of variability, particularly the two prominent peaks between residues 67–77 and 79–86, and the variability in the N-terminal region also (amino acids 10–15). This may be significant because in immunoglobulins, these three regions are clustered together on the outside of the binding site and are thought to be involved in some sort of second-site interaction¹². A fourth peak of variability, noted by Patten *et al.*¹², which is internal to the immunoglobulin V structure, is much less prominent than observed previously but still seems significantly more variable than the equivalent region of V_H (or V_{α} , data not shown). A note of caution in this interpretation is raised by the analyses of Barth *et al.*¹³ who found somewhat less variability in a plot of 10 V_{β} sequences, particularly in the 65–86 amino-acid range.

Although the analysis is somewhat flawed by the failure to compare identical numbers of V_H and V_β sequences, it does illustrate the varied results which can be obtained with the sequences available.

We conclude that V_β and probably V_α sequences all share the three major regions of variability exhibited by immunoglobulins and that this is another indication of the similarity of their structures. There exist additional regions of variability in the N-terminal portions of V_α and V_β and between residues 67 and 86 in V_β that seem qualitatively different from equivalent regions of immunoglobulins, but these must be considered somewhat speculative at present. It is interesting, however, that Sim and Augustin (ref. 37 and personal communication) have found mutations in a β -chain gene which may alter the specificity of a T_H hybridoma and that some of these occur in these additional regions of variability. This type of data, together with site-directed mutagenesis, may provide the direct functional link between changes in T-cell specificity and these regions of variability that will be necessary to resolve some of these issues.

One striking feature of mouse V_β gene segments is that most of those in the literature (5/6)^{12,13,38} occur as single-copy elements in the genome, in contrast to mammalian V_κ ^{39,40} or V_H ⁴¹ sequences which occur as large (4–60) cross-hybridizing families. Another apparent difference between V_β and immunoglobulin V segments is the more rapid rate of divergence between species in the former compared with the latter¹². A third distinguishing feature of V_β sequences is the fact that a relatively small number of sequences make up the bulk of the utilized repertoire in the thymus, which, together with the lack of cross-hybridizing gene families, suggests a limited repertoire of utilizable V_β sequences¹². Hood and colleagues have extended this finding to T-cell lines and hybrids and estimate that there are fewer than 21 distinct V_β gene segments provided the usage is relatively random¹³. Southern blot analysis of five of the seven distinct murine V_α families described in the present paper (Fig. 4) indicates that all of these occur as families of closely related sequences in the genome. It also seems from Fig. 4 that there will be many more V_α than V_β gene segments. Specifically, each of the five V_α genes surveyed in Fig. 4 hybridizes to between four and nine discernible bands in mouse DNA. *EcoRI* digests were done as well as the *HindIII* analysis shown, and in each case a total of 35–40 distinct bands are visible with these probes. Together with the four bands seen with the HDS58 V-region⁹ probe and the six observed with MD13-10 (data not shown), there appears to be a minimum of 45–50 V_α gene segments. Using the 30% occurrence of pseudogenes in mouse immunoglobulin V regions⁴¹, this sets the V_α repertoire at a minimum of 30 functional sequences and possibly more. Despite this similarity with immunoglobulin V regions in terms of cross-reactive families, the data in Fig. 4 also indicate that V_α s are diverging almost as quickly between species as V_β s. This contrasts with the multiple, strongly cross-reactive bands seen in very distant species for several V_H ⁴⁸ probes and a V_κ ⁴² probe using the same criterion (as well as the unusual C5 V_β family¹²). We conclude that while V_α and V_β sequences are under different selective pressures with respect to cross-reactive families and repertoire size, they diverge from each other at similar rates.

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Abrogation of IL-3 and IL-2 dependence by recombinant murine retroviruses expressing v-myc oncogenes

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Several oncogenes are thought to cause transformation by affecting the signal transmission pathway of growth factors. One example is the induction of c-myc, the cellular homologue of the avian transforming oncogene v-myc, by platelet-derived growth factor (PDGF) among a set of genes associated with competence induction in fibroblasts^{1,2}. Another of the competence genes, r-fos, has been shown to be related to v-fos, the transforming gene of the FBJ sarcoma virus³. In addition, PDGF induces c-fos, the cellular homologue of v-fos^{4–6}. The importance of c-myc induction is suggested by the observation that c-myc, under the control of a

glucocorticoid regulator, can partially relieve the requirement of fibroblasts for PDGF⁷. We have examined the effects of oncogenes on haematopoietic/lymphoid cell differentiation, immortalization and factor dependence for growth. Here we report the effects of recombinant murine retroviruses capable of expressing the avian *v-myc*. With interleukin-3 (IL-3)- or interleukin-2 (IL-2)-dependent cells, the viruses abrogated the requirement for growth factors and suppressed *c-myc* expression.

IL-2 is required for the proliferation of antigen-activated mature T cells (see ref. 8 for review) whereas IL-3 induces the proliferation and differentiation of early haematopoietic/lymphoid stem cells (see refs 9, 10 for review). Several IL-2-dependent cytotoxic T-cell lines have been established. The frequency of establishing IL-2-dependent lines suggests that an immortalizing event is required¹¹. In our experiments we used a simian virus 40 (SV40)-specific, H-2K^b-restricted, cytotoxic T-cell line (CTB6) from C57BL/6J mice¹² (provided by Dr Barbara Knowles).

IL-3-dependent cell lines have been isolated from primary retrovirus-induced lymphomas^{13,14} and from long-term bone marrow cultures^{15,16}. In the latter case an immortalizing event may also be required. We used the FDC-P1 cell line which has properties of early myeloid lineage cells^{15,17}. These cells have a normal diploid karyotype and differ from normal IL-3-responsive cells in their limited capacity to differentiate.

To evaluate the effects of *v-myc* expression, we used constructs of avian *v-myc* oncogenes in murine retroviruses (Fig. 1). J-2 and J-3 viruses are derivatives of the 3611 murine sarcoma virus (MSV)¹⁸, which contains the *v-raf* oncogene¹⁹; the MH2-transforming virus²⁰, which contains the avian homologue of *v-raf*, *v-mil*^{21,22} and the *v-myc* oncogene; and of the acute avian leukaemia virus MC29 which contains only the *v-myc* oncogene^{23,24}. The J-2 virus contains a hybrid *v-raf/v-mil* oncogene and a hybrid *v-myc* derived from MH2 and MC29 and includes the splice acceptor sequences present in the 5' portion of MH2 *v-myc*²². To evaluate the contribution of *v-raf/v-mil*, a derivative (J-3) with a 200-base deletion was used which shifts the *v-raf/v-mil* oncogene out of the reading frame. The deletion eliminated the fibroblast-transforming activity and expression of the *v-raf* protein (data not shown). An additional construct (HF) was used in which the MC29 *v-myc* oncogene was cloned into the *gag* gene of Moloney leukaemia virus (MoLV) (K.B. and H. Fan, in preparation).

The biological activity of the J-2 recombinant virus was initially evaluated as it could be assayed by transforming activity

on fibroblasts^{25,26}. When either the FDC-P1 or the CTB6 cells were exposed to the virus and maintained in IL-2 or IL-3, <0.001% of the cells were infected. This resistance was observed with both MoLV ecotropic and amphotropic helper viruses (data not shown) and has been described for cultured T cells²⁷. In the absence of IL-3, FDC-P1 cells rapidly lose viability and factor-independent variants have not been obtained^{15,17,28}. However, when cells were exposed to the J-2 virus and cultured in the absence of IL-3, factor-independent cells were obtained. Factor-independent lines were not obtained with FDC-P1 cells alone or with cells exposed to the helper virus. When the factor-independent cells were assayed, transforming virus was detectable (data not shown). Similarly, when CTB6 cells were exposed to the J-2 virus and cultured in the absence of IL-2, factor-independent cell lines were obtained which replicated transforming virus.

Previous studies have demonstrated that transformation of haematopoietic cells with the 3611 MSV^{25,29,30} does not abrogate the requirement for IL-3 for growth. To evaluate the contribution

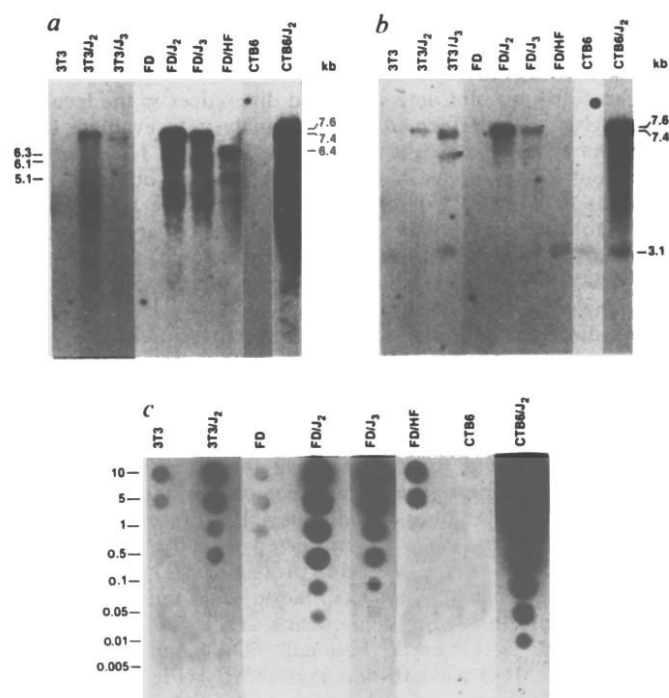


Fig. 2 Northern analysis of recombinant retroviruses in factor-independent cell lines. Polysome-associated RNA was prepared as described⁴⁹ and poly(A) RNA was selected by two cycles of oligodeoxythymidylic cellulose chromatography. The RNA (10 µg) was denatured by glyoxalation⁵⁰, separated electrophoretically in 1.2% agarose, blotted onto nitrocellulose (Schleicher and Schuell), and hybridized under stringent conditions⁵¹ with ³²P-labelled nick-translated⁵² DNA probes. **a**, *v-myc* hybridizations. For a *v-myc* probe, the 1.4-kb *Pst*I/*Aha*III fragment (Oncor Inc.) of the MC29 provirus was used^{23,24}. The intensity of hybridization between the various samples varied considerably; lanes 1–3 and 8 were exposed for 3 days whereas lanes 4–7 and 9 were exposed for 20 h. Recombinant retroviral genome-sized RNAs are indicated at the right; subgenomic RNAs are indicated at the left. Sizes are given in kb and were determined from ³²P-labelled, denatured *Hind*III fragments of bacteriophage λDNA. **b**, *raf* hybridizations. The blot shown in **a** was stripped⁵¹ and hybridized as above with 2.9-kb human *raf* cDNA probe (T. Bonner *et al.*, in preparation). The exposure of all samples was 3 days. The 3.1-kb transcript is the endogenous *raf* mRNA. **c**, Quantitative analysis of *v-myc* RNA levels in factor-independent cell lines. Poly(A) RNA, isolated from the indicated cell lines, was denatured by treatment with formamide/formaldehyde⁵¹ and loaded onto wells of a dot-blot apparatus (Schleicher and Schuell). The amount of poly(A) RNA (µg) in each well is indicated at the left margin. Hybridizations were done with the *v-myc* probe and blots were exposed for 20 h.

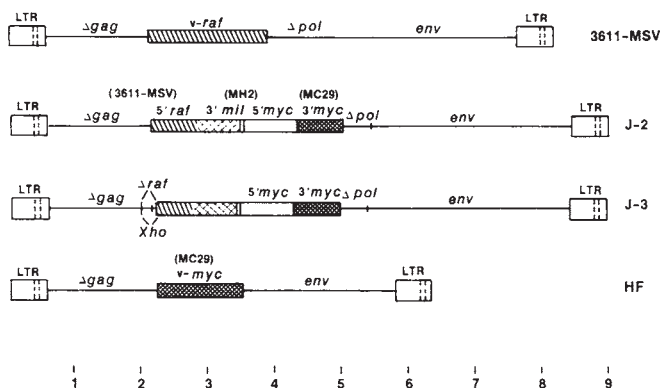


Fig. 1 Genomic organization of the defective 3611-MSV and of the *v-myc*-containing recombinant viruses. The origin of the specific *v-onc* sequences in the constructs is indicated by: ▨, 3611-MSV *v-raf*; ▤, MH2 *v-mil*; ▥, MH2 *v-myc*; ▦, MC29 *v-myc*. The double bars 5' of the MH2 *v-myc* in the J-2 and J-3 viruses indicate the position of the splice acceptor sequences for the *v-myc* gene. The sizes of the viruses (bottom line) are shown in kilobases. LTR, long terminal repeat. The construction and the production of the recombinant J-2 and J-3 viruses^{25,26} and the HF virus (K.B. *et al.*, in preparation) have been described.

of the *v-raf/v-mil* oncogene, FDC-P1 cells were exposed to the J-3 virus or the HF virus. In both cases factor-independent FDC-P1 cell lines were obtained when cells were cultured in the absence of IL-3.

Representative lines were examined for *v-raf/v-mil* and/or *v-myc* RNAs by Northern blot analysis (Fig. 2). In uninfected NIH 3T3, FDC-P1 or CTB6 cells there were no detectable *v-myc* hybridizing RNAs (Fig. 2a), whereas there was a 3.1-kilobase (kb) RNA detected with the *raf* probe (Fig. 2b). This RNA has the expected size of the endogenous *c-raf* messenger RNA and was observed at comparable levels in all infected cell lines. The 7.6- and 7.4-kb genomic RNAs of the J-2 and J-3 viruses, respectively, were detectable using *v-myc* and *raf* probes in infected NIH 3T3 cells, in FDC-P1 cells infected with the J-2 or J-3 viruses or CTB6 cells infected with the J-2 virus. With the *v-myc* probe, major subgenomic RNAs of 6.3 and 6.1 kb for the J-2 and J-3 viruses, respectively, were also observed. The precise origins of these RNAs have not been defined. In FDC-P1 cells infected with the HF virus there was a major 6.4-kb RNA which hybridized with the *v-myc* probe but not with the *raf* probe, consistent with the genomic structure of the virus. In addition there was a major subgenomic RNA of 5.1 kb whose origin is not known. Similar anomalous transcripts have been observed with other recombinant retroviruses³¹.

The Northern blot data suggested differences in the levels of viral RNA. We performed dot blot analysis to evaluate these differences. FDC-P1 cells infected with J-2 or J-3 virus had ~10-fold higher *v-myc* hybridizing RNA than observed with fibroblasts (Fig. 2c). CTB6 cells infected with the J-2 virus had ~100-fold higher RNA levels. These differences are not caused by multiple integrated proviruses (data not shown) and therefore may be the result of lineage-specific effects on enhancer sequences³².

We also examined the cell lines for expression of the *v-myc* and *v-raf/v-mil* proteins. All the lines examined expressed the *v-myc* protein of relative molecular mass (M_r) 62,000 at levels comparable to those of *c-myc* in the parental FDC-P1 cells. Because of the cross-reactivity of the anti-*myc* sera with mouse *c-myc* protein and the similarity in the sizes^{33,34}, the identification of the protein as viral relies on the observation that none of the infected lines contain *c-myc* RNA (see below). With either FDC-P1 or CTB6 cells infected with the J-2 virus the expected *gag-(v-raf/v-mil)* fusion protein of M_r 75,000 (ref. 18) was detectable by immunoprecipitation (data not shown).

To evaluate the factor dependence, ³H-thymidine incorporation assays were used. Parental FDC-P1 cells required ~0.2 ng ml⁻¹ IL-3 for half-maximal activity (Fig. 3a). In contrast, J-2, J-3 or HF virus-infected cells showed no IL-3 dependence. Similarly, the J-2-infected CTB6 cells showed a vastly decreased requirement for IL-2 (Fig. 3b). The growth curves and the doubling times were comparable for each of the virus-infected FDC-P1 cell lines in the absence of IL-3 and for the parental cell line grown in the presence of IL-3 (Fig. 3c).

One mechanism for the abrogation of factor dependence is the production of a requisite growth factor. However, none of the infected cell lines produced a mitogenic activity for the parental cell lines (data not shown). In the assay for IL-3, the lack of detectable activity indicates concentrations of <~0.02 ng ml⁻¹. We also examined the effect of an antiserum³⁵ against IL-3. The immune IgG inhibited the IL-3-dependent proliferation of the parental FDC-P1 cells but had no effect on J-2, J-3 or HF virus-infected cells (Fig. 3d).

The absence of a requirement for IL-3 suggested that the expression of IL-3 receptors is altered, therefore we determined the binding of cells to ¹²⁵I-labelled IL-3. In previous studies³⁶ the FDC-P1 cells have been shown to express ~2,000 receptors per cell with an apparent K_d of 1.7×10^{-11} (ref. 36). The FDC-P1 cells infected with the J-2, J-3 or the HF virus bound iodinated IL-3 at levels comparable to the uninfected parental cells (data not shown).

The retention of receptors for IL-3 raised the question of whether IL-3 could alter the expression of *c-myc*. We detected

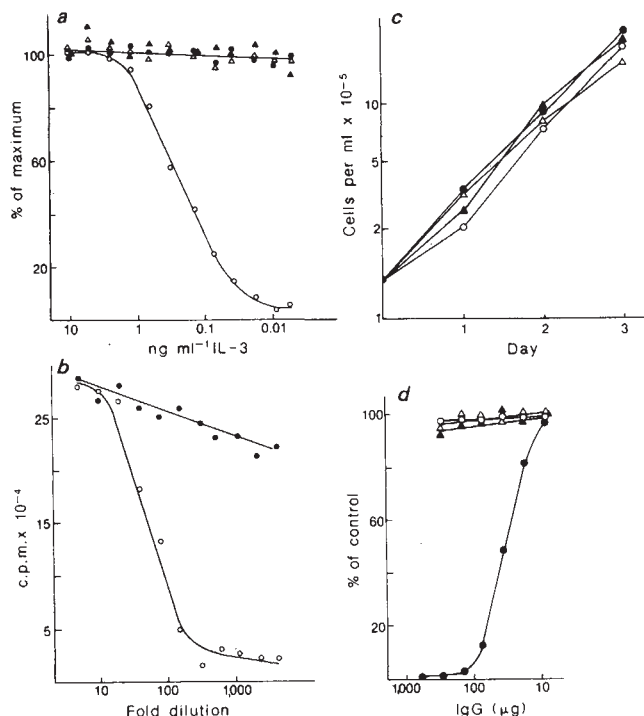


Fig. 3 Proliferative responses of various cell lines to IL-3 or IL-2.

a, The indicated cells were obtained from exponentially growing cultures and were pelleted and resuspended twice in RPMI 1640-containing 10% fetal calf serum (FCS). In 96-well microtitre plates appropriate dilutions of purified IL-3 were made in wells containing 0.05 ml of RPMI 1640 with 10% FCS to which 0.05 ml containing 10^5 cells were added. The cells were incubated for 24 h at 37 °C after which 1 μ Ci of ³H-thymidine was added per well and the cells incubated for an additional 6 h at 37 °C. The cells were collected and IL-3 purified to apparent biochemical homogeneity as described previously⁵³. Results are plotted as the percentage of maximal ³H-thymidine incorporation seen in the presence of excess IL-3. The cell lines examined included the parental FDC-P1 cells (○—○) and FDC-P1 cells infected with the J-2 (●—●), J-3 (△—△) or HF (▲—▲) viruses. **b**, As **a** except cells were incubated for 48 h before pulsing with ³H-thymidine. IL-2 was partially purified from conditioned media from induced EL-4 cells as described previously⁵⁴. The cells examined included the parental CTB6 cells (○—○) and CTB6 cells infected with the J-2 virus (●—●). **c**, Growth curves for control and infected FDC-P1 cells obtained by seeding the cells at 5×10^5 cells per ml in RPMI 1640 containing 10% FCS in the presence or absence of IL-3. Viable cell numbers were determined at the indicated times. The cells examined included the parental FDC-P1 cells (○—○) which were grown in the presence of IL-3 (20 units ml⁻¹) or J-2 (●—●), J-3 (△—△) or HF (▲—▲) infected FDC-P1 cells which were grown in the absence of IL-3. **d**, Lack of inhibition of growth by antisera against IL-3. The indicated cells were obtained from exponentially growing cultures and were pelleted and resuspended twice in RPMI 1640-containing 10% FCS. In 96-well microtitre plates, twofold dilutions of immune or control, protein A-Sepharose-purified IgG were made in 0.05 ml RPMI 1640-containing 10% FCS. For the parental FDC-P1 cells, 0.01 ml IL-3 (2 U) was added to each well. The samples were incubated for 2 h at 37 °C and the cells (10^5) in 0.05 ml were added. The cells were incubated for 24 h at 37 °C and pulsed with ³H-thymidine as in Fig. 4. The cell lines examined included the parental FDC-P1 cells (●—●) and J-2 (○—○), J-3 (▲—▲) and HF (△—△) infected FDC-P1 cells. The preparation and characteristics of the immune IgG have been described previously³².

c-myc RNA in the parental FDC-P1 cells grown in IL-3, but no *c-myc* in the virus-infected cell lines grown in the presence or absence of IL-3. To determine whether this was caused by low levels under steady-state conditions, cells were examined at various times after exposure to IL-3. No *c-myc* RNA was detected at 30 min, 2 or 72 h in culture (Fig. 4b). To determine whether the absence of *c-myc* RNA was a general property of cells

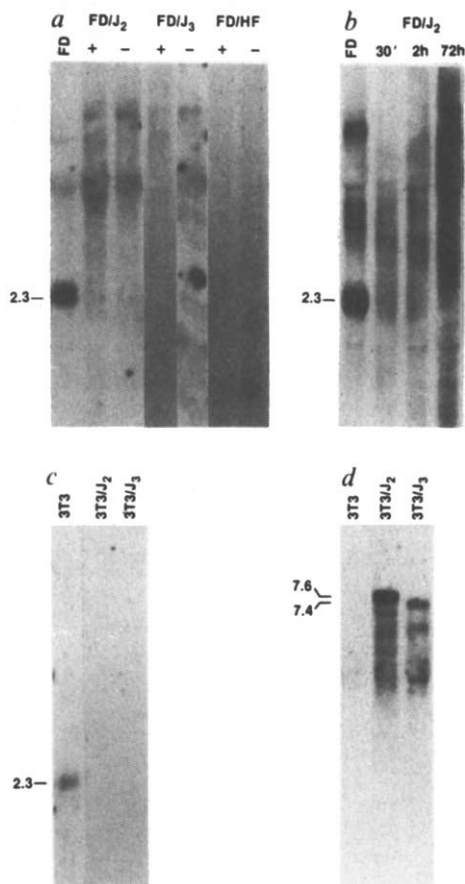


Fig. 4 *a*, *c-myc* expression in FDC-P1 lines in the presence (+) and absence (-) of IL-3. Total RNA was prepared⁵⁵ and poly(A)-containing RNA was selected by oligo(dT) cellulose chromatography. The RNA (15 µg) was denatured, electrophoresed, blotted and hybridized as described in Fig. 2. The *c-myc* probe was the 5.6-kb *Bam*HI fragment of the mouse genomic *c-myc*⁴⁰. *b*, Time course examining *c-myc* expression. Total RNA was prepared from FDC-P1 (J-2) cells at the indicated times (in h) after the addition of IL-3 and poly(A) RNA was analysed as above. The autoradiographs were overexposed to detect *c-myc* transcripts. *c*, *d*, *myc* expression in NIH 3T3 lines. Poly(A) RNA (15 µg) was prepared from the indicated cell lines and analysed by Northern blot hybridization. *c-myc* hybridizing is shown in *c* in which exposure of the autoradiograph was for 1 week. *v-myc* hybridization is shown in *d* and the exposure of the autoradiograph was for 3 days.

infected with these recombinant viruses, we examined the cloned, producer-fibroblast cell lines. We readily detected *c-myc* RNA in control fibroblasts but not in infected cells (Fig. 4c).

We next determined whether the abrogation of a requirement for IL-3 affected tumorigenicity. The J-3- and HF-infected cells were tested for tumour formation in *nu/nu* mice, as neither of these viruses induced tumours during the time they were examined²⁶. The parental FDC-P1 cells were not tumorigenic, as described previously¹⁵, although FDC-P1 cells infected with the J-3 or the HF virus induced tumours (data not shown).

The ability of *v-myc* expression to abrogate the requirement for IL-3 or IL-2 supports the hypothesis that these factors regulate proliferation through their effects on *c-myc* expression. This conclusion is consistent with previous studies indicating that PDGF induction of proliferation of fibroblasts is mediated by its ability to induce *c-myc* expression⁷. Therefore, we speculate that a common pathway for growth regulation exists that is characterized by growth-factor induction of *c-myc* expression.

Surprisingly, we were unable to induce *c-myc* expression with IL-3 in the infected cells. The absence of *c-myc* in these cells is probably caused by an effect of *v-myc* on *c-myc* expression as suggested by the observation that infected fibroblasts, or J-2 transformed pre-B cells (data not shown), had no detectable

c-myc RNA. The specific suppression of *c-myc* expression is suggested by the lack of a comparable effect on *c-ras* expression. The inability to induce *c-myc* is not caused by alteration of the endogenous loci as determined by Southern blot analysis of *Eco*RI-restricted DNAs (data not shown). The ability of an activated *c-myc* locus to negatively regulate the normal allele has been suggested by other studies³⁷⁻⁴². The mechanisms for this effect are not known but may involve the interaction of the *myc* protein or a *myc*-induced protein with potential regulatory sequences defined by sensitivity to DNase I (ref. 42). Experiments are in progress to assess the DNase I sensitivity of these sites in the *c-myc* loci of infected FDC-P1 cells.

The absence of *c-myc* expression in haematopoietic/lymphoid cells or fibroblasts infected with the *v-myc* constructs used here contrasts with recent studies⁴³ in which Rat-1 cells, carrying a transfected *myc* oncogene, expressed *c-myc* in tissue culture. The basis of the differences is not known, although they may be caused by differences in the levels of expression of the introduced *myc* oncogenes. In the studies of Keath *et al.*, the transfected *myc* RNA was expressed at levels that were 2-6-fold higher than that of the endogenous gene, whereas in our studies the levels of *v-myc* RNA were ~100-fold higher than normal *c-myc* levels in fibroblasts.

We have examined the ability of other oncogenes to affect IL-3 dependence. The 3611 transforming virus, Harvey sarcoma virus and MoSV have been shown to immortalize haematopoietic cells without altering the requirement for IL-3 for growth^{25,29}. This suggests that some oncogenes affect different components of growth regulation, for example, commitment and cell-cycle progression. The concept that *v-ras* and *v-myc* affect different cell-cycle components may be important for understanding the synergy between these two oncogenes for tumour induction²⁶ and for transformation of primary bone marrow cells (E. Blasi, L. Varesio and U.R.; J.I. and U.R., in preparation).

Abelson murine leukaemia virus has been shown to abrogate the requirement for IL-3 for growth⁴⁴. As with *c-myc*, factor independence is not caused by an autocrine-type mechanism. The possibility that the *abl* oncogene functions through the regulation of *c-myc* expression is suggested by the constitutive expression of *c-myc* in Abelson MuLV-transformed, IL-3-independent cells. We are currently evaluating FBR MSV as, under appropriate conditions, IL-3 induces *c-fos* transcription (K. Kelly and J.I., unpublished data). Preliminary results however, suggest that FBR MSV does not abrogate the requirement for IL-3.

Tumorigenesis requires multiple events. In myeloid tumours, stages of tumour progression have been described that affect differentiation and growth-factor dependence^{45,46}. For example, murine myeloid leukaemias are blocked in differentiation but require IL-3 for growth^{13,14}. Similarly, HTLV-1-induced cutaneous T-cell lymphomas require IL-2 growth^{47,48}. In these cases, the activation of *c-myc* can be envisioned as a mechanism for progression to factor-independent growth. A similar role for *c-myc* activation may occur in other lineages, such as B cells, where there is a high incidence of altered *c-myc* expression associated with transformation.

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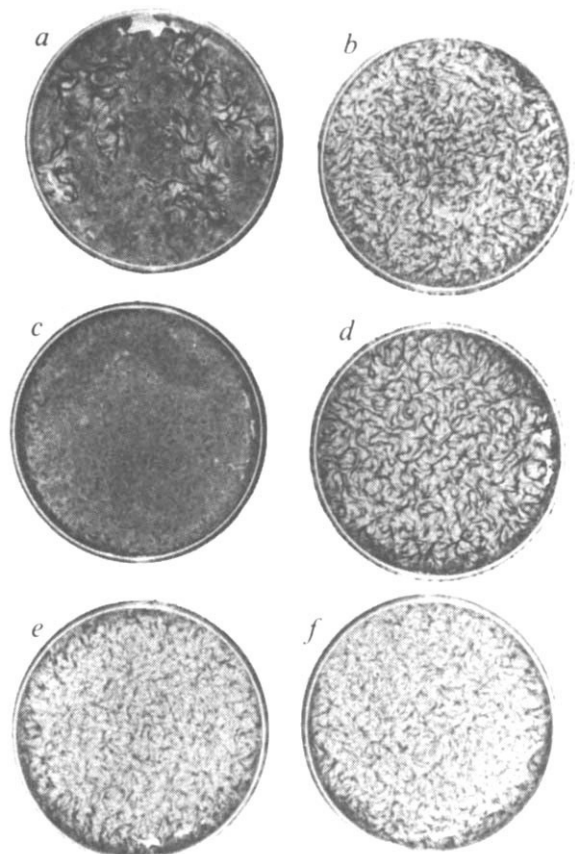


Fig. 1 SSV-transformation of human fibroblasts. *a*, 20 days after infection with 20 FFU per dish of SSV/SSAV; *b*, 20 days after infection with 2,500 FFU per dish of SSV/SSAV; *c*, control culture 21 days after plating; *d*, culture grown for 20 days with 100 ng ml⁻¹ PDGF; *e*, culture grown for 20 days with 100 ng ml⁻¹ EGF; *f*, same as *e* but with the addition of 500 µg anti-PDGF IgG per ml. **Methods.** SSV/SSAV (SSV-1, lot 24-51) purified from A204-SSV-1 cells, was obtained from Pfizer Inc., Maywood, New Jersey. 7.0×10^4 virus particles from this preparation corresponded to 1 FFU in an acute transformation assay on human foreskin fibroblasts, AG 1523 (from the Human Genetic Mutant Cell Repository, Camden, New Jersey), performed as follows. Cells were plated sparsely (10^4 cells cm⁻²) in Eagle's MEM supplemented with 10% NCS (Gibco). On the following day, cultures were exposed to SSV/SSAV for 1 h, followed by a medium change. Subsequently, fresh medium was given every second day and cultures were continuously examined for the presence of foci. Fixation after the appropriate time was in methanol/acetic acid (3:1) followed by staining with Giemsa. PDGF was purified as described elsewhere¹⁷. EGF (receptor grade) was purchased from Collaborative Research. Representative samples from six independently performed experiments are shown.

Antibodies against platelet-derived growth factor inhibit acute transformation by simian sarcoma virus

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A clue to the molecular mechanism of neoplastic transformation was provided by the finding of a near identity in amino-acid sequence between the platelet-derived growth factor (PDGF) B-chain and a region in the transforming protein, p28^{sis}, of simian sarcoma virus (SSV)¹⁻⁴, an agent that causes sarcomas and gliomas in experimental animals^{5,6}. This finding infers a direct link between the molecular biology of normal mitogenesis and oncogenesis since it suggests that the transforming activity of SSV is caused by a growth factor. Although PDGF agonist activity has been isolated from conditioned medium of SSV-transformed cells⁷⁻¹⁰, it is not clear whether infection of responsive cells by SSV leads solely to autocrine stimulation of growth by a secreted PDGF-like factor or whether other, possibly intracellular, activities of p28^{sis} or its processed products contribute to the transformation^{9,11,12}. To distinguish between these possibilities, we have studied the effect of anti-PDGF antibodies on acute SSV-transformation, and report here that these antibodies inhibit both proliferation and SSV-induced morphological changes in human diploid fibroblasts.

Previous studies on the biosynthesis, processing and biological activity of p28^{sis} have been performed on SSV-transformed cells in long-term culture⁷⁻¹². For our purposes, such cell lines are

of limited value as transformed cells in continuous culture are likely to undergo secondary phenotypic changes that are unrelated to the expression of the primary transforming oncogene (compare with ref. 13). Therefore, we have chosen to study the effect of anti-PDGF antibodies on acute SSV-transformation of normal human diploid fibroblasts.

The human foreskin fibroblast line AG 1523 was readily transformed by SSV/simian sarcoma-associated virus (SSAV). Following infection with a low virus titre (20 focus-forming units (FFU) per dish), foci of transformed cells could be identified under the microscope after ~4 days, and were macroscopically visible after ~8 days. Foci were sharply demarcated and consisted of parallel arrays of densely packed hyperchromatic fusiform cells (Fig. 1*a*). In the case of infection with high titres of SSV/SSAV (2,500 FFU per dish), individual foci could no longer be seen; rather, the normal swirled growth pattern of the fibroblast cultures was exaggerated and the cells appeared in multi-layered ridges (Fig. 1*b*). A similar effect was seen when

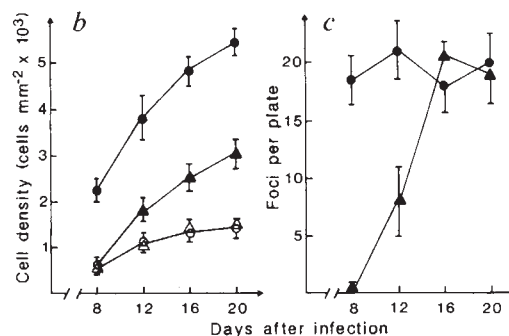
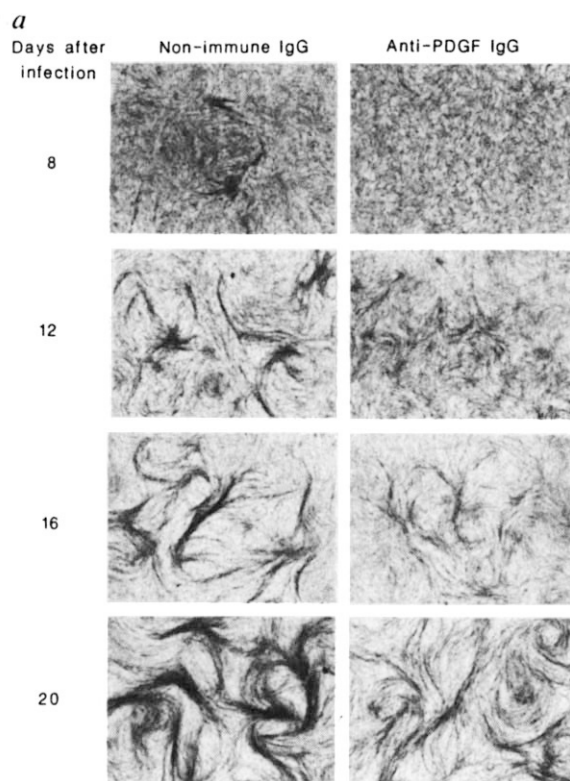


Fig. 2 *a-c*, Effect of anti-PDGF IgG on SSV-induced focus formation. *a*, Morphology of foci. Photomicrographs of representative fields in cultures treated with anti-PDGF IgG and non-immune IgG for one of four independently performed time-course experiments (summarized in *c*) are shown. *b*, Cell density in foci. The effect of anti-PDGF IgG (▲) and non-immune IgG (●) on the cell density of individual foci at various times after SSV/SSAV infection was compared with the effect of anti-PDGF IgG (Δ) and non-immune IgG (○) on the cell density of a non-transformed fibroblast monolayer. The cell number was counted in five squares ($8.1 \times 10^{-3} \text{ mm}^2$) in each of seven foci per plate. Values are mean $\pm$ s.d. for four separate experiments. *c*, Number of visible foci in SSV-transformed cultures treated with anti-PDGF IgG (▲) and non-immune IgG (●) at various times after infection. Values are mean $\pm$ s.d. for four independent experiments.

Methods. Infection was carried out as described in Fig. 1 legend using 20 FFU per dish. Fresh medium supplemented with anti-PDGF antibody¹⁵ ($500 \mu\text{g ml}^{-1}$) or non-immune IgG ($500 \mu\text{g ml}^{-1}$) [IgG fractions were obtained by chromatography on protein A-Sepharose (Pharmacia)] was added immediately after infection and changed every second day. Cultures were fixed and stained at various times after infection.

the fibroblasts were grown in the presence of PDGF or epidermal growth factor (EGF) (Fig. 1*d, e*). Thus, the morphology of both the individual SSV-transformed AG 1523 cells (SSV-1523) and the extensively transformed culture was indistinguishable from that obtained by exogenous growth factors. The appearance of an individual focus seems to reflect the normal growth pattern of human fibroblasts and is compatible with a local action of a growth factor.

As the *v-sis* gene product is structurally, functionally and immunologically related to PDGF^{1-4,7-12}, we investigated whether SSV-transformation could be inhibited by neutralizing the secreted *v-sis* product with anti-PDGF antibodies. Such inhibition was observed in the presence of high concentrations ($500 \mu\text{g ml}^{-1}$) of anti-PDGF antibodies but not with immunoglobulins from non-immune sera (Fig. 2). Suppression of focus formation was most evident early after infection with SSV/SSAV. At day 8, when foci became macroscopically visible in the presence of non-immune IgG, no foci at all could be detected in the presence of anti-PDGF antibody (Fig. 2*a, c*). At day 12, foci became visible in anti-PDGF antibody-treated cultures, but were less conspicuous than in controls (Fig. 2*a*). With increasing time after infection and increased cell density, it became evident that neither the number of foci (Fig. 2*c*) nor the area (Fig. 2*a*) of individual foci was affected by anti-PDGF antibodies. Rather, the cell density (Fig. 2*b*) in the focus was affected, suggesting suppression of the growth of the transformed cells. The suppressive effect of anti-PDGF antibodies on focus formation was independent of the number of FFUs used for infection, nor was it affected by the concentration of fetal calf serum used (not shown).

One explanation as to why complete suppression of focus formation was seen only early after infection, when a confluent layer of cells was not yet formed (see Fig. 1 legend for experimental details), is that the interaction between the PDGF-like factor and the anti-PDGF antibodies is sterically restricted in dense cultures. Experimental evidence in support of this assumption was obtained when dense cultures of cells transformed by

high titres of SSV/SSAV (Fig. 1*d*) were trypsinized and plated sparsely; subsequent ³H-thymidine labelling in serum-free medium revealed a fourfold decrease in the fraction of labelled nuclei in the presence of anti-PDGF IgG compared with cells treated with non-immune IgG (Table 1). The fraction of labelled nuclei in anti-PDGF antibody-treated cultures (6.7%) was comparable to the fraction in non-transformed cultures of AG 1523 cells in the same conditions (Table 1). In addition, the anti-PDGF IgG caused a phenotypic reversion; in the presence of antibodies, SSV-1523 cells had a flat, normal appearance (Fig. 3). A nonspecific effect of the anti-PDGF antibodies on cell growth and morphology was unlikely as the stimulatory effect of EGF on cell proliferation and morphology (Fig. 1*f*) as well as growth in monolayer culture (Fig. 2*b*) was unaffected. Furthermore, a nonspecific effect of anti-PDGF antibodies on primary infection and spreading of SSV/SSAV is unlikely as neither the number of visible foci (Fig. 2*c*) nor focus area

Table 1 Effect of anti-PDGF IgG on ³H-thymidine incorporation in SSV-1523 cells

Cells	% Labelled nuclei	
	Non-immune IgG	Anti-PDGF IgG
AG 1523	4.0 $\pm$ 1.0	5.3 $\pm$ 1.5
SSV-1523	27.3 $\pm$ 2.9	6.7 $\pm$ 1.0

Twenty-four days after infection of a 10-cm^2 culture of AG 1523 cells with 2,500 FFU SSV/SSAV, the cells were trypsinized and plated sparsely (10^4 cells cm^{-2}) in Eagle's minimal essential medium (MEM), 10% newborn calf serum (NCS). One day later, the medium was changed to MCDB 104, with the Ca^{2+} concentration reduced to 0.5 mM^{16} . At the same time, either anti-PDGF IgG ($500 \mu\text{g ml}^{-1}$) or non-immune rabbit IgG ($500 \mu\text{g ml}^{-1}$) was added; 24 h later, the cells were labelled with ³H-thymidine (Amersham; final concentration $0.5 \mu\text{Ci ml}^{-1}$, 5 Ci mmol^{-1}) for 48 h followed by fixation and autoradiographic analysis as described elsewhere¹⁶. Values are mean $\pm$ s.d. for six measurements in two separate experiments.

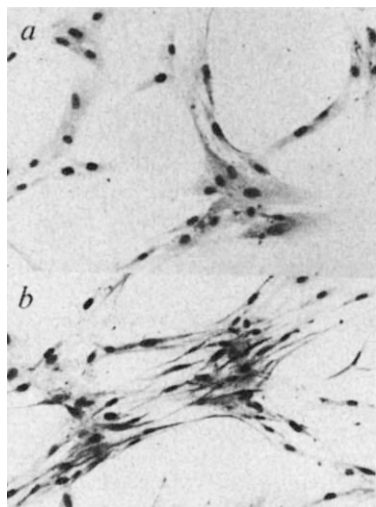


Fig. 3 Effect of anti-PDGF IgG on sparse SSV-1523 cells. Photomicrographs of representative fields of autoradiographs from cultures of sparse SSV-1523 cells treated with anti-PDGF IgG (a) or non-immune rabbit IgG (b). For experimental procedure see Table 1 and ref. 16.

(Fig. 2a) was impaired. Clearly, therefore, it is possible to suppress the expression of the transformed phenotype in human SSV-transformed fibroblasts by specific neutralization of a released factor by antibodies to PDGF.

PDGF receptor-competing activity was undetectable in medium conditioned by cells early (12–20 days) after infection with 20 FFU per dish of SSV/SSAV (not shown). However, medium from extensively transformed cultures (2,500 FFU per dish) contained receptor-competing activity equivalent to 5 ng ml⁻¹ of PDGF; this activity was neutralized by a 2-h incubation with 10 µg ml⁻¹ anti-PDGF IgG, which is comparable to the concentration of antibody required to neutralize 5 ng ml⁻¹ of authentic PDGF (not shown). The requirement of higher concentrations (500 µg ml⁻¹) of anti-PDGF IgG to suppress SSV/SSAV-induced focus formation in conditions in which the overall concentration of PDGF-like activity is ≤ 5 ng ml⁻¹ of PDGF, gives further support for a steric restriction, perhaps due to the juxtacellular diffusion boundary layer¹⁴ which may hinder efficient interaction between the antibodies and the secretory *v-sis* product in dense cultures.

We have posed the question of whether SSV-induced transformation consists exclusively of autocrine growth stimulation, mediated by an externalized *v-sis* product, or whether additional and possibly intracellular activities of that product contribute to the expression of the transformed phenotype. In support of the latter hypothesis, Huang and co-workers reported recently that anti-PDGF antibodies partially inhibit growth but do not revert transformation of certain SSV-transformed cell lines⁹; their view was based on results using SSV-transformed counterparts of continuous cell lines (NIH 3T3, NRK and marmoset fibroblasts). However, such cell lines frequently acquire spontaneous genotypic and phenotypic changes; as a result, after long-term culture their transformed properties become unrelated (to an unknown extent) to the activity of the *v-sis* gene *per se*. We therefore chose to study primary transformed cultures of human diploid fibroblasts (a cell type in which spontaneous transformation does not occur); in this system anti-PDGF antibodies inhibit both proliferation and SSV-induced morphological changes, suggesting that SSV-transformation is due solely to the autocrine action of a PDGF agonist. It might be argued that only a paracrine action of an externalized *v-sis* product was inhibited by anti-PDGF antibodies, leaving the transformed cells unaffected. However, the outstretched and sharply demarcated appearance of the SSV foci is inconsistent with an extensive paracrine stimulation of neighbouring cells, in which situation the presence of more diffuse foci would be expected.

Moreover, in sparse SSV-1523 cells derived from extensively transformed cultures, anti-PDGF antibodies inhibited ³H-thymidine incorporation to control levels and reverted the morphological expression of transformation. We therefore conclude that the anti-PDGF antibodies neutralized a secreted PDGF-like growth factor(s), the autocrine action of which is essential for SSV-induced transformation.

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Amplification and altered expression of the *c-myc* oncogene in A-MuLV-transformed fibroblasts

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Chromosomal rearrangements involving the *c-myc* oncogene are a prevalent feature of plasmacytomas that arise after inoculating BALB/c mice with pristane and Abelson murine leukaemia virus (A-MuLV)^{1–3}. With this observation in mind, we decided to determine if any genetic alterations of the *c-myc* locus could be observed in cells of a different type, when transformed *in vitro* by A-MuLV. Here we have analysed three independent A-MuLV-transformed NIH 3T3 lines (ANN-I, 54c12 and N25), and found that the *c-myc* locus is amplified 8–19-fold in each transformant. Quantitative S₁ nuclease mapping performed on ANN-I and 54c12 RNAs demonstrated that: (1) *c-myc* messenger RNAs accumulated to double the levels found in NIH 3T3 cells; and (2) a shift in the use of the two normal *c-myc* transcription initiation sites (P₁ and P₂) occurred in favour of the 3' site, P₂. Analysis of *c-myc* chromatin by DNase I treatment of 54c12 nuclei revealed that most, if not all, of the *c-myc* gene copies were transcriptionally competent. We present alternative ideas to explain why amplification of the *c-myc* gene occurs repeatedly in A-MuLV-transformed fibroblasts. Finally, we discuss our results in relation to the hypothesis linking the phenomenon of tumour progression with the amplification of oncogenes.

The transformed cell lines ANN-I, 54 and N25 were originally derived by infection of NIH 3T3 cells *in vitro* with different strains of A-MuLV^{4–6}. ANN-I and N25 are non-producer lines which respectively carry one genome of the A-MuLV(P120) strain and two genomes of the A-MuLV(P90) strains^{4,5}. 54c12 has been derived from the non-producer cell line N54 by superin-

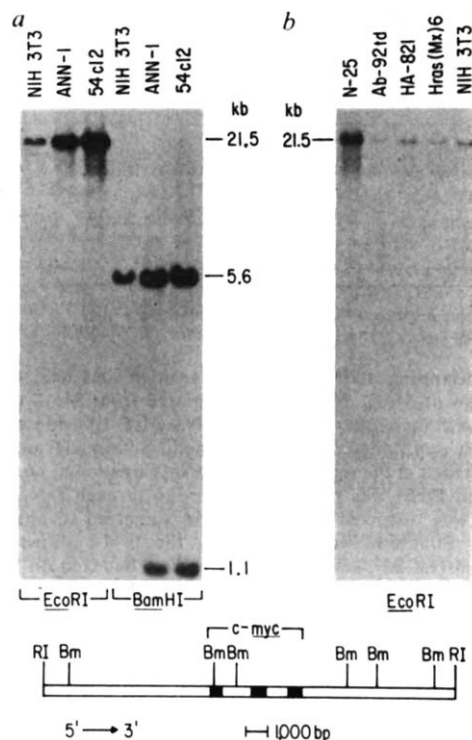


Fig. 1 Southern blot analysis of the *c-myc* loci in various cell lines. Genomic DNAs (7.5 μ g) from the indicated cell lines were digested with either *Eco*RI (RI) or *Bam*HI (Bm), electrophoresed on a 0.7% agarose gel and transferred to nitrocellulose filters. The resulting blots were hybridized³² with a murine *c-myc* complementary DNA probe: a 1.9-kb *Hind*III fragment obtained from the pMc-myc54 cDNA clone which contains most of exons 1 and 3 and all of exon 2 (ref. 8). The blots were exposed for 17 h (a) and 6 h (b), with an intensifying screen. Fragment sizes are indicated and the copy numbers of the *c-myc* genes are presented in Table 1. 54c12, ANN-I and N25 are NIH 3T3 cell lines transformed with the A-MuLV(P160), A-MuLV(P120) and A-MuLV(P90) strains, respectively⁴⁻⁶. 92td is a NIH 3T3 cell infected with A-MuLV(P92td), a transformation-defective mutant⁷. HA-821 is an NIH 3T3 cell line transformed with the HA-821 strain of Harvey murine sarcoma virus³³. Hras(mx)6 is an NIH 3T3 line transformed by HrasNVX, a Moloney murine retroviral vector containing a neomycin selection gene³⁴ and the Harvey murine sarcoma virus oncogene³⁵ (L. W. Stanton and K.B.M., unpublished results). A restriction map of the *c-myc* locus is shown at the bottom: the three *c-myc* exons are indicated by solid bars.

fection of the latter with Moloney murine leukaemia virus (N. Rosenberg, personal communication); it produces particles of the A-MuLV(P160) strain^{5,6}. The A-MuLV(P120) differs from A-MuLV(P160) in that the former has an 800-base-pair (bp) deletion in the *v-abl* portion of the gag-abl polyprotein⁶. The genome of A-MuLV(P90) is similar in size to A-MuLV(P120) but produces a gag-abl polyprotein of relative molecular mass 90,000, presumably as the result of early translational termination⁶.

DNA samples from these three A-MuLV transformants were analysed by Southern hybridization using a *c-myc* cDNA probe. Digestion of NIH 3T3 DNA with *Eco*RI produced a *c-myc*-containing fragment of 21.5 kilobases (kb) that extended ~8 kb 5' and 3' of the *c-myc* gene. This 21.5-kb *c-myc* band was considerably more intense in ANN-I, 54c12 and N25 than in the NIH 3T3 control (Fig. 1a, b). On the other hand, there was no evidence of *c-myc* amplification in two NIH 3T3 lines transformed by Harvey *ras* retroviruses or in an NIH 3T3 line harbouring the genome of a transformation-defective A-MuLV strain, P92td (ref. 7) (Fig. 1b). *Bam*HI digestion of NIH 3T3 DNA produced two hybridizing bands of 5.6 and 1.1 kb. The smaller fragment, which was barely discernible in the NIH 3T3

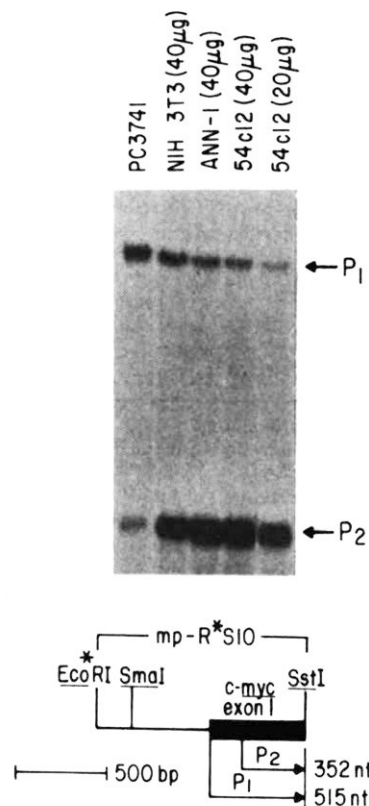


Fig. 2 Relative expression of two normal *c-myc* gene promoters (P_1 and P_2) in NIH 3T3 and A-MuLV-transformed NIH 3T3 cell lines ANN-I and 54c12 (see Fig. 1). Total cytoplasmic RNAs were hybridized to a uniformly labelled, single-stranded DNA probe encompassing *c-myc* 5'-flanking and exon 1 sequences, mpR*S10 (ref. 13), exhaustively digested with S_1 nuclease and then analysed on a 7 M urea polyacrylamide gel. The location of S_1 -protected bands corresponding to normal *c-myc* gene promoters 1 and 2 are indicated. The amounts of total *c-myc* mRNAs as well as the relative utilization of P_1 and P_2 are presented in Table 1. PC3741 is an NZB plasmacytoma¹³.

lane in Fig. 1a, was very intense in ANN-I and 54c12. Quantitative comparison of these *c-myc* signals by densitometer analysis revealed 8-, 13- and 19-fold *c-myc* amplifications in N25, ANN-I and 54c12, respectively.

We next determined whether amplification of the *c-myc* gene resulted in a parallel increase in *c-myc* mRNAs. *c-myc* transcripts are normally derived from two transcriptional start sites (P_1 and P_2) which are separated by 163 bp at the 5' end of a noncoding first exon⁸⁻¹¹. Total RNA was extracted¹² from ANN-I, 54c12 and NIH 3T3 cell lines and subjected to quantitative S_1 nuclease analysis with a uniformly labelled, single-stranded DNA probe (mpR*S10 in Fig. 2) encompassing most of the *c-myc* first exon and 550 bases of 5'-flanking DNA¹³. S_1 nuclease-resistant bands corresponding to transcripts derived from P_1 and P_2 were apparent in all three cell lines (Fig. 2). The results of densitometric tracings of these S_1 -protected bands are presented in Table 1 and may be summarized as follows. (1) Despite their amplified *myc* loci (13-19-fold), total amounts of *c-myc* mRNAs are only slightly elevated in ANN-I (1.6-fold) and 54c12 (2-fold) compared with NIH 3T3 cells; (2) this augmentation of *c-myc* mRNA levels is totally accounted for by an increase of the mRNAs initiated at P_2 (1.9- and 2.5-fold in ANN-I and 54c12 cells, respectively); (3) remarkably, the amounts of P_1 -derived transcripts are diminished 1.5- and 2-fold in ANN-I and 54c12, respectively. We conclude that in ANN-I and 54c12 cells a shift in the use of the two *c-myc* promoters has occurred which favours P_2 . The steady-state levels of these mRNAs may reflect the rate of utilization of the two *c-myc* promoters, as the

Table 1 *c-myc* gene copy number and expression in various cell lines

Cell line	<i>c-myc</i> gene copy no.*	Amounts of <i>c-myc</i> mRNAs (relative to NIH 3T3 cells)†	P ₁ P ₂ ‡	Retroviral strains‡
NIH 3T3	1	1	0.33	
54c12	19	2	0.07	A-MuLV(P160)
ANN-I	13	1.6	0.12	A-MuLV(P120)
N-25	8	ND	ND	A-MuLV(P90)
Ab92td	1	ND	ND	A-MuLV(P92td)
HA-821	1	ND	ND	MuSV(HA-821)
Hras(mx)6	1	ND	ND	HrasNVX

* Expressed per haploid genome.

† Data obtained from densitometric tracings of Fig. 2. ND, not determined.

‡ Described in Fig. 1 legend.

stabilities of these transcripts were found to be comparable in different cellular contexts¹⁴.

In all but one of the tumour cell lines where amplification of the *c-myc* gene has previously been reported, the level of *c-myc* mRNA corresponded to the gene copy number¹⁵⁻¹⁹. The exception was a human gastric adenocarcinoma cell line, SC-2, composed of a mixture of undifferentiated cells and differentiated, non-proliferating mucous epithelial cells¹⁹. In view of this cellular heterogeneity, the authors proposed that the lower than expected level of *c-myc* transcripts in SC-2 cells could reflect the dual nature of this line, with the actively dividing undifferentiated cells producing most, if not all, the *myc* RNAs. Clearly, such an explanation could not be involved for the ANN-I and 54c12 cell lines. The unexpectedly low expression of the amplified *c-myc* genes in ANN-I and 54c12 cells could mean that not all of these genes are transcriptionally competent. For example, most of the *c-myc* copies could reside in closed chromatin domains which are transcriptionally inactive. One way to assess this possibility is to look for the presence of DNase I-hypersensitive sites, which have been correlated with *c-myc* gene expression in human²⁰ and murine cells (P.D.F., unpublished results). Figure 3 shows that with increasing amounts of DNase I, a *Kpn*I fragment spanning 10 kb of the *c-myc* locus is gradually degraded, while several smaller fragments are generated. Further mapping with different restriction enzymes and DNA probes allowed us to determine that these smaller fragments are produced by DNase I cleavage at the three major hypersensitive sites, I, II and III²⁰. Densitometric analysis of this autoradiogram revealed that most (>70%) of the *c-myc* copies in 54c12 contain these DNase I-hypersensitive sites. We conclude that the relatively low expression of the amplified *c-myc* genes cannot be ascribed to the inaccessibility of *c-myc* templates embedded in inactive chromatin. However, a direct comparison of *c-myc* transcription rates will be required to investigate further the novel mechanism(s) for *c-myc* control in these A-MuLV transformants.

Thus, in three independent fibroblast cell lines transformed *in vitro* with different strains of A-MuLV, the *c-myc* oncogene is amplified, resulting in quantitative and qualitative changes in its expression. The three A-MuLV strains, A-MuLV(P160), A-MuLV(P120) and A-MuLV(P90), contain the *v-abl*-specific sequences that have been demonstrated to be sufficient for protein kinase activity and the transformation of fibroblasts²¹⁻²³. Another fibroblast cell line, carrying a genome of the non-transforming strain A-MuLV(P92td)⁷, shows no *c-myc* gene amplification; the same is true for two fibroblast cell lines transformed by retroviruses containing a different transforming gene, the *ras* oncogene (see Fig. 1b). We interpret these results as meaning that in stably transformed fibroblast cell lines, specifically those containing an A-MuLV transforming gene, the amplification of *c-myc* is at least a frequent if not a constant event.

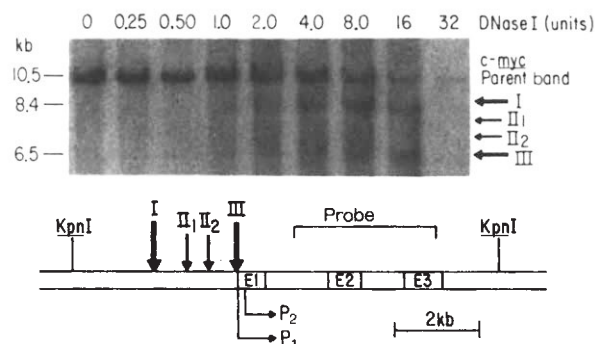


Fig. 3 Mapping of DNase I-hypersensitive sites near *c-myc* in chromatin of 54c12. Nuclei were prepared from 54c12 cells and treated with increasing amounts of DNase I (P-L) as described by Siebenlist *et al.*²⁰. Each sample contained 2×10^7 nuclei and DNase I as indicated at the top. Following DNase treatment, nuclei were lysed and DNA was prepared. A portion of each DNA sample (10 μ g) was restricted with *Kpn*I and subjected to analysis by genomic Southern blotting³². Using the probe shown above, p25BH3.4 (ref. 32), several sub-bands were detected on DNase I treatment of chromatin from 54c12. These sub-bands correspond to those shown previously to arise from DNase I-hypersensitive regions in the 5'-flanking region of transcriptionally active human *c-myc*²⁰. These results were confirmed with different restriction enzymes and DNA probes. Boxes labelled E1, E2 and E3 represent the three exons of *c-myc*; arrows labelled P₁ and P₂ show transcriptional initiation sites for the two promoters of the gene⁸⁻¹¹.

Several models of tumorigenesis are based on the idea that increased expression of a proto-oncogene allow cells to escape normal growth controls *in vivo*²⁴⁻²⁶. One obvious mechanism to achieve elevated expression of a cellular gene is through amplification. Indeed, there is considerable evidence linking gene amplification to the generation and/or progression of a tumour²⁷⁻³¹. Here we report that three independent cell lines already transformed by the *v-abl* oncogene and maintained under no apparent selective pressure *in vitro*, carry an amplification of a second oncogene, *myc*. Amplification of an oncogene is not necessarily limited to the process of tumour progression *in vivo*. This may be because selective pressures of the same kind that occur *in vivo* also take place during growth *in vitro*. It is possible that in a population of A-MuLV-transformed fibroblasts in culture, a rare cell, in which *c-myc* activated by amplification, acquired a growth advantage. Alternatively, the frequent association between *v-abl* and the activation of the *c-myc* oncogene in murine plasma cell tumours and in fibroblasts transformed *in vitro* by A-MuLV may be the consequence of some undefined aspect of the oncogenic state induced by *v-abl*. Further experiments will assess whether the activation of *c-myc* actually confers a growth advantage on the *v-abl*-transformed cells or whether a more subtle link exists between *v-abl* and the genetic changes occurring at the *c-myc* locus.

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c-myc gene is transcribed at high rate in G₀-arrested fibroblasts and is post-transcriptionally regulated in response to growth factors

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There is increasing evidence that at least some of the cellular homologues to retroviral oncogenes (*c-onc* or proto-oncogenes) are directly linked to the control of cell growth (for a review see ref. 1). Among these, *c-myc*, the cellular homologue to the avian myelocytomatosis virus (MC29) oncogene, has been shown to express high levels of mRNA during early G₀/G₁ phase after mitogenic stimulation of T lymphocytes² by concanavalin A or of fibroblasts by platelet-derived growth factor (PDGF)² or serum³. An attractive model proposed for this regulation is that the *c-myc* gene is strongly repressed in cells arrested in the G₀ phase of the cell cycle by a growth factor-sensitive repressor⁴. We have investigated an alternative model of post-transcriptional regulation. This latter model leads to two testable predictions. First, that *c-myc* mRNA should be unusually unstable, which we have confirmed⁵. And second, that there would be a high level of constitutive expression, a situation opposite to that implied by the repressor model. Here we report that *c-myc* gene is indeed transcribed at a high rate in G₀-arrested chinese hamster lung fibroblasts, although the level of mature *c-myc* mRNA is barely detectable. The early and dramatic increase in *c-myc* mRNA levels when these resting cells are stimulated by growth factors is not accompanied by any appreciable change in the transcription rate of *c-myc* gene. Taken together these findings support a model of post-transcriptional regulation of *c-myc* expression at the level of mRNA degradation.

The occurrence of chromosomal translocations involving *c-myc* in various Burkitt's lymphoma cell lines has provided a way of comparing the expression of both alleles. The absence of detectable *c-myc* transcripts originating from the normal allele led to the conclusion that only the translocated one was expressed⁶⁻⁸. This suggested a regulation by *trans*-acting elements⁹ which in turn led to the proposal of several models

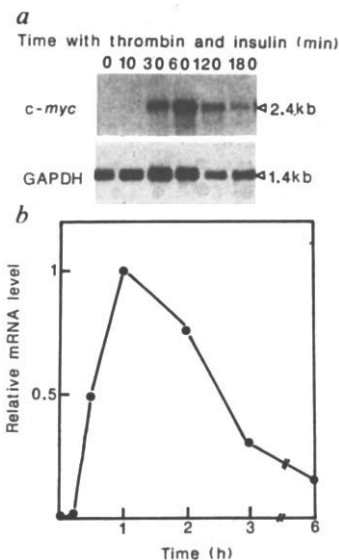


Fig. 1 Induction of *c-myc* mRNA on stimulation of quiescent CCL39 cells by thrombin with insulin.

Methods. Confluent CCL39 cells were arrested in G₀/G₁ by serum deprivation for 27 h in 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium¹⁰ and then stimulated to progress synchronously through the cell cycle by adding α -thrombin (2 U ml⁻¹) and insulin (10 μ g ml⁻¹). For each time point, 2×10^7 cells from one 14-cm Petri dish were washed twice with 10 ml ice-cold phosphate-buffered saline (PBS) and once with 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂. They were then scraped off and broken with a Dounce homogenizer in 1 ml of the above buffer containing 1% v/v Triton X-100 and 10 mM vanadyl ribonucleoside (BRL). Nuclei were spun down for 15 s in an Eppendorf centrifuge and the supernatant was digested for 2 h at room temperature with 200 μ g ml⁻¹ proteinase K after addition of one volume of 20 mM EDTA, 0.3 M NaCl, 2% w/v SDS and 20 mM Tris-HCl pH 7.5. After phenol-chloroform extraction and ethanol precipitation, poly(A)⁺ RNA was obtained according to Bantle and Maxwell²⁷. **a**, Poly(A)⁺ RNA prepared from 250 μ g total RNA was fractionated on 1.2% formaldehyde-agarose gels and transferred to nitrocellulose following the procedure of Thomas²⁸. Filters were prehybridized for 4 h in a solution containing 50% v/v formamide, 20 mM NaH₂PO₄ pH 7, 0.75 M NaCl, 5 $\times$ Denhardt's solution, 100 μ g ml⁻¹ sonicated and denatured salmon sperm DNA, 0.1% SDS and 10% dextran sulphate. Filters were hybridized for 24 h with 5×10^6 c.p.m. of ³²P-labelled nick-translated *c-myc* (pRYC 7.4)¹⁷ probe (specific activity $2-3 \times 10^8$ c.p.m. per μ g), washed at 60 °C in 0.2 $\times$ SSC, 0.1% SDS and autoradiographed for 2 days using Kodak XAR film with a Quanta III DuPont Cronex intensifying screen at -70 °C. The first probe was removed by five washes in boiling water and filters were rehybridized with the GAPDH (pRGAPDH13)¹² probe. **b**, Northern blots of **a** were quantified by densitometric scanning and *c-myc* mRNA levels were expressed as the ratio of *c-myc*/GAPDH signals.

involving a transcriptional repressor^{2,4}, although the alternative possibility of a controlled degradation of the mRNA could not be formally excluded. The control protein, be it a repressor or an element of the degradation process, was shown to be labile from the superinduction in *c-myc* mRNA levels observed after inhibition of protein synthesis². Although the repressor model could explain the sharp increase in *c-myc* mRNA in response to mitogenic stimulation, it could not account alone for a transient peak of expression occurring at a precise time of the G₀/G₁ transition unless the mRNA is rapidly degraded or somehow inactivated. Indeed, we have recently shown⁵ that *c-myc* mRNA is extremely unstable in various human cells, whether normal or transformed, thereby supporting a model of regulation through mRNA degradation. These two models lead to opposite predictions with respect to the basal level of *c-myc*

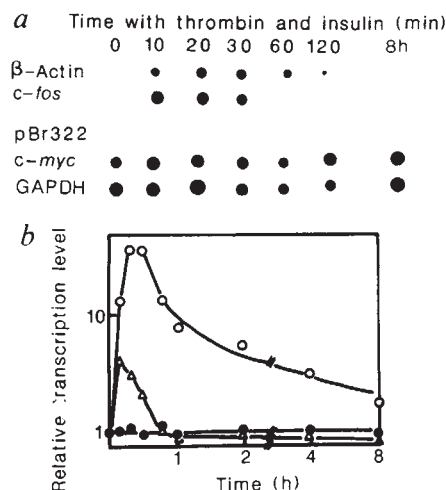


Fig. 2 Nuclear run-on analysis of *c-myc* gene activity in CCL39 cells stimulated to grow by thrombin plus insulin. Nuclei prepared from 2×10^7 CCL39 cells either G_0 -arrested or stimulated to grow for indicated times as in Fig. 1 were resuspended in 50 mM Tris-HCl pH 8.3, 40% v/v glycerol, 5 mM $MgCl_2$ and incubated for 10 min with 200 μ Ci of [α - 32 P]UTP (Amersham, 400 Ci mmol $^{-1}$) in conditions described by Schibler *et al.*¹⁶ for elongation of nascent RNA chains. Deproteinized RNA (6×10^6 c.p.m.) was then hybridized to nitrocellulose filters carrying dot-spots of 5 μ g alkali-denatured DNA from β -actin (pAL41)¹⁹, *c-fos* (pc-fos(mouse)-3)¹⁸, pBR322, *c-myc* (pRYC 7.4)¹⁷ and GAPDH (pRGAPDH13)¹². Hybridization and washing conditions were as described elsewhere⁴. Autoradiographs were exposed for 14 h for β -actin and *c-fos* and 24 h for *c-myc* and GAPDH (a). Signals were quantitated by densitometric scanning and expressed with reference to zero time (G_0 -arrested cells). The *c-myc* curve is actually a composite of five independent experiments (b). ○, β -actin; △, *c-fos*; ●, *c-myc*.

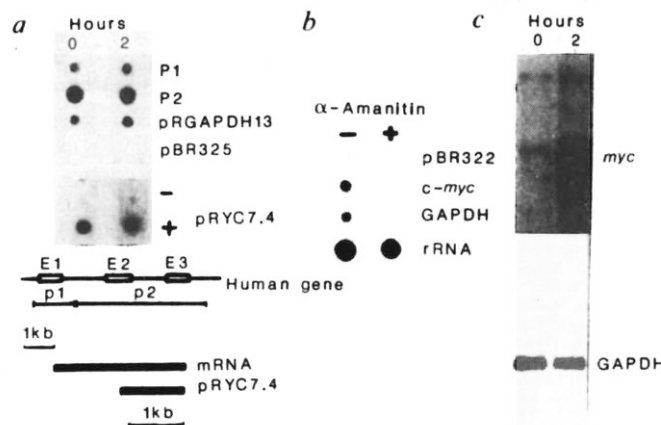


Fig. 3 Characteristics of the transcriptional activity observed in Fig. 2. **a**, 32 P-labelled RNA from *in vitro* incubated nuclei from G_0 -arrested and 2-h-stimulated cells as above was hybridized to dot-spots carrying 5 μ g of indicated DNA probes. P1 and P2 were *Xba*I/*Eco*RI subclones of the human *c-myc* genomic clone constructed and kindly donated to us by Dr D. Stehelin²⁹. Coding (+) and noncoding (−) strands of pRYC 7.4 were subcloned in M13mp9. In this latter case, the autoradiograph was overexposed to emphasize the lack of hybridization with (−) strand. **b**, 32 P-labelled RNA from nuclei from G_0 -arrested cells were incubated *in vitro* as above in the presence (+) or absence (−) of 2 μ g ml $^{-1}$ α -amanitin and hybridized to dot-spots carrying 5 μ g of indicated DNA probes. The rDNA probe contains a 6.7-kb *Eco*RI genomic DNA subfragment cloned in pBR322 (pMEB 2) and was obtained from Dr J. P. Bachellerie³⁰. **c**, The Northern blot was obtained from 0 and 2-h-stimulated cells and successively hybridized with *c-myc* (overexposed for 1 week) and GAPDH probes (14 h exposure) as in Fig. 1. In this latter case, RNA was prepared with GuSCN/LiCl²⁰ and total poly(A) $^{+}$ RNA analysed by Northern blotting.

transcription. Under the repressor hypothesis, the basal rate of transcription should be minimal and inducible^{2,4}, while the degradation model implies that *c-myc* would be constitutively transcribed at high rate. We therefore investigated the transcriptional status of the *c-myc* gene when G_0 -arrested fibroblasts are stimulated by purified growth factors.

We used a non-tumoral line of Chinese hamster lung fibroblasts (CCL39) which can be reversibly arrested in G_0/G_1 by serum growth factor deprivation¹⁰. Addition of two purified growth factors (insulin and α -thrombin) to these resting cells initiates early biochemical responses¹¹ required for subsequent DNA replication and growth. We therefore analysed the *c-myc* mRNA content in poly(A) $^{+}$ RNA by RNA blotting experiments performed at various times during growth factor-induced G_0/G_1 transition. Figure 1a shows that virtually no *c-myc* mRNA is detectable until 10 min after addition of thrombin plus insulin, when a sharp rise is seen for 1 h followed by a more gradual decline over a period of several hours. To take into account experimental variations in the amount of RNA transferred to the nitrocellulose, all densitometric quantitations were made with reference to the signals obtained by rehybridizing the same blots with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe¹² which showed no reproducible variations over several independent experiments. The resulting normalized kinetic curve is shown in Fig. 1b.

At this point, this transient accumulation can be accounted for either by a stimulation of transcription or by any post-transcriptional event (maturation, transport or stabilization of the message). The first possibility was addressed by carrying out *in vitro* nuclear run-on experiments at various times after addition of growth factors so as not to miss a transient and very early wave of transcriptional stimulation which would only materialize as cytoplasmic mRNA at later times. Recent

reports^{13–15} indeed show that such a situation does exist in the case of *c-fos*, whose transcription is greatly increased within minutes after administration of purified growth factors to quiescent 3T3 cells.

In vitro transcripts were generated by elongation of previously initiated RNA chains during incubation of nuclei isolated from cells at various stages of the G_0/G_1 transition in the presence of [α - 32 P]UTP as described by Schibler *et al.*¹⁶. In these conditions, which preclude initiation of new transcripts, this assay provides a reasonable measurement of the number of polymerase molecules actively engaged in transcription before cell lysis. Run-on transcripts were then hybridized to dot-spots of plasmid DNA containing rat GAPDH complementary DNA (pRGAPDH13)¹², *c-myc* cDNA covering the 3' half of the messenger (pRYC 7.4)¹⁷, *c-fos* genomic DNA (pc-fos(mouse)-3)¹⁸ and β -actin mouse cDNA¹⁹ as described in Fig. 2 legend. There was no significant difference between transcription rates in quiescent and stimulated cells in the cases of *c-myc* and GAPDH, while β -actin and *c-fos* exhibited a strong stimulation (Fig. 2a). Figure 2b shows the relative transcriptional level of *c-myc*, *c-fos* and β -actin after normalization of the densitometer scanings with reference to GAPDH. The same experiment was repeated five times and the resulting spots obtained with *c-myc* and GAPDH were scanned and normalized with reference to the signals obtained with resting cells. In no case did the variation observed with the *c-myc* signal exceed a factor of two. In a control experiment, run-on transcripts of nuclei from cells arrested in G_0 and stimulated for 2 h were hybridized to probes covering different regions of the gene as well as separated strands. Several controls were made (Fig. 3) to establish the validity of the above nuclear run-on data: (1) Transcription was indeed from the coding strand (left panel). (2) Furthermore, the stoichiometry observed between signals obtained with probes

covering different parts of the gene served to show that the entire gene was transcribed at the same rate, thereby excluding the possible release of an attenuation block occurring near the origin of transcription (Fig. 3a). (3) α -Amanitin ($2 \mu\text{g ml}^{-1}$) completely blocked transcription from *c-myc* and GAPDH while not affecting that of ribosomal DNA. This control, carried out on both G_0 -arrested (Fig. 3b) and 2-h-stimulated cells (not shown) with similar results, establishes that the transcripts observed are made by RNA polymerase II. A Northern blot of RNA from the same cells purposely overexposed to reveal the basal level of *c-myc* expression in quiescent cells (Fig. 3c) allowed the detection of a larger band (~ 5 kilobases; kb) which remained constant in both situations. The possibility of non-specific hybridization with rRNA has not been rigorously excluded and is under investigation using an intron probe. However, the occurrence of the same signal when using purified exon 3 probe (not shown) makes this unlikely.

The above results strongly point to a crucial, although possibly not exclusive, contribution of degradation mechanisms to the regulation of *c-myc* expression in cells stimulated to grow. In a rather similar situation, the mitogenic stimulation of quiescent 3T3 cells by serum or PDGF, Greenberg and Ziff¹³ noted a small increase in *c-myc* transcription which, as pointed out by these authors, could not by itself account for the dramatic increase in cytoplasmic *c-myc* mRNA. Should the 5-kb transcript turn out to be a real precursor, its constant level would raise the question as to whether the nuclear pre-messenger or the cytoplasmic messenger is the main target for degradation.

The comparison of transcription rates of *c-myc* and GAPDH in quiescent cells shows perhaps our most significant results. The housekeeping enzyme GAPDH is known to be abundant in nearly all tissues²⁰ as well as in undifferentiated cells like HeLa cells^{21,22} and in any event certainly more so than the *myc* protein, despite the lack of quantitative information about the latter. In sharp contrast to the relative amounts of GAPDH and *myc* proteins, the rate of nuclear transcription of the *c-myc* gene is roughly comparable to that of GAPDH (after correcting for the length of probes used). This finding clearly indicates that the *c-myc* promoter is quite active even in quiescent cells. It therefore contradicts the prediction of models for the regulation of *c-myc* expression based on a transcriptional repressor^{2,4} which implied a low basal rate of transcription⁴. The two predictions of our model of regulation at the level of messenger stability, the instability of *c-myc* mRNA and a high level of constitutive transcription, are now clearly fulfilled. In the light of this new model, it would be worth reconsidering the assumption that, in Burkitt's lymphoma cell lines where *c-myc* gene is translocated, the normal allele is not expressed⁷⁻⁹. Further experiments should aim to establish whether it is truly transcriptionally silent, as suggested by DNase sensitivity studies²³, or whether its transcripts are so unstable as to escape detection.

Recent reports have provided several examples of post-transcriptional regulation. Groudine and Casimir²⁴ have shown that the chicken thymidine kinase gene is transcribed at the same level in dividing and non-dividing cells while important variations of mRNA were observed. Cleveland and Havercroft²⁵ have shown that the tubulin mRNA content could be reduced in colchicine-treated CHO cells while transcription remained unchanged. Recent work from our own laboratory^{12,20} showed that the widely different abundance of GAPDH in various rat tissues is correlated with the abundance of its messenger while transcription rates are quite similar in all tissues. In the case of dihydrofolate reductase, Leys *et al.*²⁶ have shown the important regulatory role of nuclear transcript degradation for this gene.

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Dr M. Buckingham for β -actin, Dr J. P. Bachellerie for the rDNA probe, and Ph. Fort for fruitful discussions.

Note added in proof: We have demonstrated that the observed reduction in *c-myc* RNA in Dandi cells on treatment with interferon³¹ was due to a reduction in mRNA half-life rather than a decrease in transcription rate³². Moreover, a survey of *myc* expression in several mouse plasma cytomas suggested that post-transcriptional processes are largely responsible for the higher steady-state accumulation of truncated *c-myc* transcripts while broken and intact *c-myc* genes are transcribed at comparable rates³³.

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An unusual coding sequence from a *Drosophila* clock gene is conserved in vertebrates

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The *per* locus has a fundamental involvement in the expression of biological rhythms in *Drosophila*. Mutations at this locus can shorten, lengthen or eliminate a variety of rhythmic activities that range from circadian behaviours, exemplified by eclosion and locomotor activities^{1,2}, to short-period behaviour such as the 55-s rhythm of courtship song³. DNA from the *per* locus has been cloned⁴⁻⁷, and we have used P-element-mediated DNA transformation to establish that a 7.1-kilobase (kb) *Hind*III fragment contains a functional copy of the gene. This transforming DNA contains

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a single transcription unit which gives rise to a 4.5-kb poly(A)⁺ RNA⁵. Here we report the results of a search for sequences homologous to the *per* locus DNA in the genomic DNA of several species of vertebrates. An unusual, tandemly repeated sequence forming a portion of the 4.5-kb *per* transcript is homologous to DNA in chicken, mouse and man. Cloned DNAs from the mouse and *Drosophila* are related by long, uninterrupted tandem repetitions of the sequence ACNGGN. At the *per* locus, these tandem repeats are predicted to code for poly(Thr-Gly) tracts up to 48 amino acids long. These repeated sequences are also transcribed in the mouse. Several long tracts of poly(Thr-Gly) appear to be encoded by DNA cloned from the mouse.

A *per* locus probe was hybridized to yeast, *Drosophila*, chicken, cat, mouse and human DNA (see Fig. 1a). Strong, multiple DNA homologies were detected in the mouse, while two or three homologies were seen in *Drosophila* and chicken, and a single weaker homology was detected in human DNA. As about a dozen genomic restriction fragments in mouse DNA are homologous to the *Drosophila per* sequence, several cloned DNA segments containing *per* homology were recovered from a mouse cosmid library using the *Drosophila* 7.1-kb *Hind*III fragment as probe. Figure 1b, c shows hybridizations involving two such mouse clones, cp2.2 and cp35.3. When used as a probe, the *Drosophila* 7.1-kb *Hind*III fragment showed strong hybridization to a single restriction fragment in each mouse clone, indicating that each mouse cosmid contains a discrete region that is highly homologous to the *Drosophila* DNA. More extensive mapping of cosmid cp2.2 has localized the region of homology to a 2.5-kb *Eco*RI fragment (Fig. 1d). This mouse restriction fragment is homologous to a short region of the *Drosophila per* locus; all homology in the *Drosophila* DNA is confined to a 1-kb *Eco*RV/*Bam*HI fragment (Fig. 1d). The 2.5-kb *Eco*RI fragment from mouse cosmid cp2.2 was also used as a hybridization probe on mouse cosmid cp35.3 DNA. Restriction fragments labelled in cosmid cp35.3 by the *Drosophila* probe (compare with Fig. 1c) were also labelled by the mouse cp2.2 probe. Thus, all three clones seemed to carry a common sequence homology.

A portion of the 2.5-kb *Eco*RI fragment from mouse cp2.2 (0.5-kb *Eco*RI/*Stu*I; for location see Fig. 3 legend) was also hybridized to restriction digests of total genomic chicken, cat, mouse, rat and human DNA (Fig. 1e). As shown below, this mouse probe is composed primarily of a DNA sequence that is shared by the *Drosophila* and mouse clones. Hybridizing restriction fragments were observed in all the DNA samples tested, and the restriction fragments previously labelled with the

Drosophila probe (Fig. 1a) were again labelled with the mouse probe (Fig. 1e). Additional fragments were labelled with the mouse probe in most of the vertebrate DNA preparations. Thus, the same sequence homology should be responsible for at least the common hybridizations seen in Fig. 1a and e for DNA from chicken, mouse and man.

Figure 2a shows that the DNA sequence shared by *Drosophila* and vertebrates is transcribed in *Drosophila*. The 2.5-kb *Eco*RI fragment from mouse cp2.2 was hybridized to poly(A)⁺ RNA from Oregon R, a wild-type strain of *Drosophila*, and we found three size classes of poly(A)⁺ RNA (5, 4.5 and 3.5 kb) that are homologous to the mouse probe. No hybridization to the 4.5-kb poly(A)⁺ transcript was detected in RNA derived from aneuploid flies of the genotype *Df*(1)TEM202/*Df*(1)64j4 (not shown), which are specifically deficient for the transcription unit encoding the 4.5-kb *per* RNA⁵. Therefore, it can be concluded that the 4.5-kb RNA detected with the mouse probe in Oregon flies is the *per* transcript. The 5-kb and 3.5-kb transcripts hybridizing with the mouse probe are apparently transcribed from regions of the *Drosophila* genome carrying short *per* locus homologies, as described previously⁴⁻⁶.

Figure 2b shows that the 2.5-kb *Eco*RI fragment from the mouse clone cp2.2 is homologous to discrete mouse poly(A)⁺ transcripts. All mouse tissues examined produced homologous RNA, including brain, intestine, liver, spleen, kidney and a myeloma cell line. As these hybridizations were carried out at very high stringency (see Fig. 2 legend), the transcripts detected must be extensively homologous to cp2.2. Figure 2c shows that additional mouse transcripts are detected with less stringent hybridization conditions; this result suggests that more than one member of the family of *per*-homologous repeat DNA sequences is transcribed in the mouse. This notion is supported by Fig. 2d, which shows that many of the mouse transcripts detected in Fig. 2b and c are also seen when labelled *Drosophila per* locus sequences are used as a probe. As mouse and fly probes hybridize to common RNAs, we conclude that a DNA sequence shared by the two species is transcribed in the mouse.

The complete DNA sequence of the mouse 2.5-kb *Eco*RI fragment contained in cp2.2 is presented in Fig. 3. The DNA sequence of the 1-kb *Eco*RV/*Bam*HI fragment of the *Drosophila per* locus has also been determined, and the mouse-homologous region of this *Drosophila* DNA, which is just less than 0.20 kb, is presented in Fig. 4. The *Drosophila* sequence consists of a tandemly repeated hexamer which can be represented as (ACNGGN)_x. Thus, 24 tandem copies of this sequence are found, without interruption, at the *per* locus. The ACNGGN

Fig. 1 DNA homologous to the *Drosophila per* locus is present in vertebrates. **a**, Total genomic DNA from yeast (0.5 µg), *Drosophila* (0.5 µg), chicken (5 µg), cat (10 µg), mouse (10 µg), and human (10 µg) was digested with *Bam*HI, subjected to electrophoresis on a 0.75% agarose gel, and transferred to a nitrocellulose filter. A 1.3-kb *Pst*I/*Bam*HI fragment from *per* was nick-translated and used as a hybridization probe (for location of probe, see **d** below). Hybridization was at 40 °C in 5×SSC, 50% formamide, 7 mM Tris pH 7.5, 1×Denhardt's solution, 25 µg ml⁻¹ salmon sperm DNA, and 10% dextran sulphate. Washes were in 0.2×SSC, 0.1% SDS at 45 °C. Numbers indicate sizes in kb. **b**, **c**, Mouse cp2.2 and cp35.3 DNAs were digested with *Bam*HI, subjected to electrophoresis, and transferred to nitrocellulose as in **a**. Blots were hybridized with nick-translated *per* 7.1-kb *Hind*III fragment (see **d** below), and washed as in **a**. **d**, Map of *per* 7.1-kb *Hind*III fragment (left) and a 6-kb *Bam*HI fragment from cp2.2 bearing homology to *per* (right). Hatched regions indicate cross-hybridizing restriction fragments. The *per* 1.3-kb *Pst*I/*Bam*HI fragment used as a probe in **a** includes the 1.0-kb *Eco*RV/*Bam*HI fragment shown in **d**. H, *Hind*III; B, *Bam*HI; R, *Eco*RI; E5, *Eco*RV; P, *Pst*I. **e**, Total genomic DNA from the indicated species (10 µg DNA from rat) was treated as in **a** except that the nitrocellulose blot was hybridized with a 0.5-kb *Stu*I/*Eco*RI fragment from mouse clone cp2.2. The DNA sequence of this probe is indicated in Fig. 3 legend. Hybridizations to mouse and rat DNAs are shown twice so that a long exposure of the human DNA hybridization can be presented. Numbers in **a**–**c** and **e** indicate sizes, in kb, of some hybridizing DNA segments as measured against restriction fragments of λ c1857 DNA.

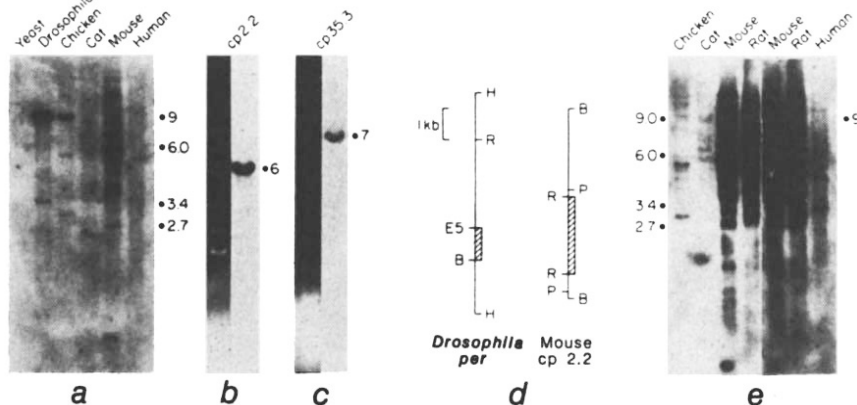
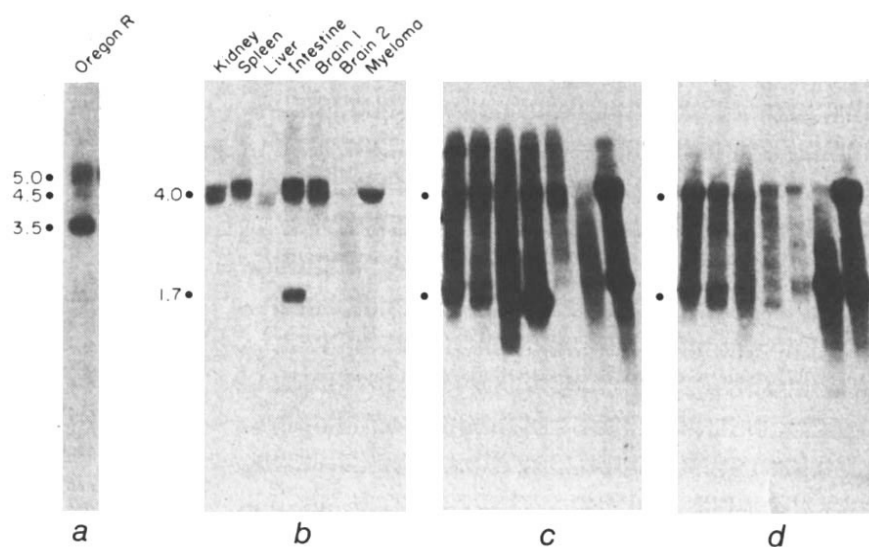


Fig. 2 Mouse/fly sequence homologies are transcribed. **a**, Poly(A)⁺ RNA (5 µg) from Oregon R flies was electrophoresed through a 1% agarose/2.2 M formaldehyde gel and transferred to a Biodyne A membrane (Pall Ultrafine Filtration). The 2.5-kb *Eco*RI fragment from mouse cosmid cp2.2 was cloned into the Riboprobe vector pSP64 (Promega Biotec) in both orientations. Single-stranded ³²P-labelled, 2.5-kb RNA probes were synthesized. In both constructs the RNA was initiated at the SP6 promoter of the vector and terminated at a *Bam*HI site in the polylinker. Only one of these two probes (that predicted to be complementary to the 4.5-kb *per* transcript), hybridized with poly(A)⁺ RNA from *Drosophila*. This probe also hybridized with mouse poly(A)⁺ RNA (see below). In **a** this probe labels three *Drosophila* transcripts of 5, 4.5 and 3.5 kb, after hybridizing at 65 °C in 50% formamide, 50 mM NaPO₄ pH 7, 0.8 M NaCl, 1 mM EDTA, 0.1% SDS, and washing at 65 °C in 5 mM NaCl, 2 mM NaPO₄ pH 7, 0.01 mM EDTA, 0.01% SDS. **b**, As in **a**, except that poly(A)⁺ RNAs (5 µg per sample) are from the indicated mouse tissues. Brain 1 is from a fetal mouse (day 16); brain 2 is from a 1-day-old mouse. All other tissues are from adults. **c**, Hybridizations to mouse transcripts were as described in **a**: washes were performed at room temperature in 5 mM NaCl, 2 mM NaPO₄ pH 7, 0.01% SDS, 0.01 mM EDTA. **d**, The mouse probe was stripped from the RNA blot used in **b** and **c** and the blot was subsequently treated as in **c** except that a Riboprobe vector carrying *per* locus DNA was used to synthesize a 2.5-kb, single-stranded, ³²P-labelled probe extending from the *per* *Hind*III site to the *Eco*RV site (see Fig. 1d). Numbers alongside each autoradiograph indicate sizes, in kb, measured against single-stranded RNA standards transcribed from restriction fragments of known lengths inserted in Riboprobe vectors.



repetition extends over a longer region, covering a total of 177 base pairs (bp), but insertions of unrelated sequences are found. These insertions consist of three base pairs or multiples of three base pairs.

In Fig. 4 the ACNGGN repeat sequence for *per* is also aligned according to DNA homology with a corresponding sequence in the mouse 2.5-kb *Eco*RI fragment. The homologies are extensive; mouse and fly sequences are composed of the common repeating hexamer ACNGGN. Although the largest stretch of uninterrupted tandem repetition is found in the *Drosophila* DNA (24 copies of the hexamer), several uninterrupted tandem repeats, each composed of 10–20 copies of the hexamer, occur in the mouse clone so that ACNGGN repeats extend with multiple interruptions over a 1.7-kb interval. In total, 28 copies of the ACNGGN hexamer are found at the *per* locus in *Drosophila* and 192 copies of the hexamer are found in the mouse clone cp2.2. The cp2.2 clone probably carries the longest run of this repetitive sequence in the mouse because the restriction map of cp2.2 corresponds to that of the most intensely labelling, *per*-homologous DNA in the mouse genome (compare with Fig. 1a).

As described earlier (Fig. 2), the tandemly repeated sequence is transcribed at the *Drosophila per* locus. Structural analyses of the 4.5-kb *per* transcript have been used to determine the size and sequence organization of several RNA coding regions at the *per* locus, and a complete report of this work will be presented elsewhere. The RNA coding region carrying the *Drosophila* ACNGGN repeat contains only one open reading frame and the predicted translation product of the hexamer repeat is poly(Thr-Gly).

In the mouse, the ACNGGN repeat sequence is part of a 2.1-kb open reading frame (Fig. 3), and is bordered by sequences resembling consensus splice acceptor (for example, position 153) and donor (for example, position 2,121) signals⁸. Each alternative reading frame carries a minimum of five stop codons in the 2.1-kb interval. The predicted translation product of the 2.1-kb open reading frame resembles that found in *Drosophila*; wherever it appears in the cloned mouse DNA, the hexamer repeat would code for poly(Thr-Gly). The hexamer repeat sequence never occurs outside the 2.1-kb open reading frame (Fig. 3), and inversions, or DNA insertions or deletions that might shift the reading frame of the hexamer from ACNGGN

to NACNGG or GNACNG, do not occur. Insertions of three base pairs or multiples of three base pairs occur often. These sequence constraints make it probable that the mouse DNA is used to encode a poly(Thr-Gly) protein segment.

In the same translational frame of the mouse DNA sequence, a second repeat unit is found that is composed of the hexamer TCAGGC; this would code for a Ser-Gly amino-acid repeat. This occurs most often in the form (Ser-Gly)₂. However, one related repeat segment includes 11 uninterrupted copies of the TCAGGC hexamer (position 1,896), and repeats of intermediate length are seen. A Ser-Gly amino-acid repeat is similar, chemically, to a Thr-Gly repeat; serine and threonine differ only by a methyl group and both are hydroxylated, uncharged, polar amino acids. Both amino acids can be phosphorylated or glycosylated. In *Drosophila*, a region beginning about 100 bp upstream of the Thr-Gly repeat is rich in Ser-Gly codons. The sequence Ser-Gly (indicated by asterisks in Fig. 4) occurs five times in this interval, with three of the *Drosophila* Ser-Gly repeats occurring within a 27-bp region coding only for these two amino acids.

The degree of sequence conservation within the mouse repeat is high. The ACNGGN repeat is almost always ACAGGC. GGT and GGG are occasionally used in place of GGC, but ACA is invariant. This degree of sequence conservation is often seen in DNA coding for proteins with long, tandemly repeated amino-acid segments^{9–13}. Proper regulation of synthesis of a protein might depend on restrictive codon usage. Alternatively, the structure of such DNA segments may be preserved by DNA conversions occurring between repeating units of the tandem array^{14–16}. A comparable level of sequence conservation is not seen in the *Drosophila* homologue (Fig. 4).

Sequence analysis of cloned mouse DNA and the *Drosophila per* locus has revealed a region of high homology based on the repeat sequence ACNGGN. ACNGGN repeat homologies have not been detected in a computer search of the GenBank and Dayhoff DNA and protein sequence libraries. However, Ser-Gly repeats are found in certain proteoglycans where they form substrates for glycosylation¹⁷. As pointed out above, the mouse and *Drosophila* clones analysed hybridize to a common set of restriction fragments derived from the total genomic DNAs of chicken, mouse and man, so the ACNGGN repeat should be responsible for all of these homologies. In contrast to the result

Fly P	Gly	Thr	Cys	Val	Ser	***	Gly	Ala	Ser	Gly	Pro	Met	Ser	Pro	Val	His	
Fly D	GGC	ACG	TGT	GTC	AGT	GGC	GCC	AGT	GGT	CGG	ATG	AGT	CCC	GTC	CAC		45
Fly P	Glu	Gly	Ser	Gly	Gly	Ser	Gly	Ser	Ser	Gly	Asn	Phe	Thr	Thr	Ala		
Fly D	GAG	GGC	AGC	GGG	GGC	AGT	GGC	TCC	TGG	GGC	AAC	TTC	ACC	ACC	GCC		90
Fly P	Ser	Asn	Ile	His	Met	Ser	Ser	Val	Thr	Asn	Thr	Ser	Ile	Ala	Gly		
Fly D	AGT	AAC	ATA	CAC	ATG	AGC	AGT	GTC	ACA	AAT	ACG	AGC	ATC	GCC	GGC		135
Mouse D								GTC	ACA	...	...	...	...	...	GGC		821
Fly P	Thr	Gly		Gly	Thr					Gly	Thr	Gly	Thr	Gly	Thr		
Fly D	ACT	GGT	ACA	GGC	ACG	...	...	...	...	GGC	ACT	GGT	ACA	GGT	ACA		165
Mouse D	ACA	GGG	ACA	GGC	ACA	GCC	AAA	GTC	ACA	GGC	ACA	GGG	ACA	GGC	ACA		866
Fly P	Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr			Gly		
Fly D	GGT	ACT	GGA	ACT	GGA	ACT	GGA	ACC	GGG	ACA	GGA	ACT	...	...	GGA		204
Mouse D	GGC	ACA	GGC	ACA	GGC	ACA	GGC	ACA	GGC	ACA	GGC	ACA	GAC	ACA	GGC		911
Fly P	Thr	Gly	Thr					Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr		
Fly D	ACC	GGG	ACA	...	...	...	...	GGA	ACT	GGA	ACC	GGG	ACA	GGA	ACT		237
Mouse D	ACA	GGC	ACA	GCC	AAA	GTC	ACA	GGC	ACA	GGC	ACA	GGC	ACA	GGT	ACA		956
Fly P	Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr	Gly	Thr	Gly		
Fly D	GGA	ACG	GGA	ACA	GGT	ACA	GGC	ACA	GGC	ACA	GGC	ACT	GGA	ACA	GGC		282
Mouse D	GGC	ACA	GGC	ACA	GGC	ACA	GGT	ACA	GGC	ACA	GGC	ACA	GGC	ACA	GGC		1,001
Fly P	Asn	Gly	Thr	Asn	Ser					Gly	Thr	Gly	Thr				
Fly D	AAT	GGA	ACA	AAT	TCC	...	...	...	...	GGC	ACC	GGA	ACC				309
Mouse D	...	...	...	...	...	GCC	AAA	GTC	ACA	GGC	ACA	GGC	ACA				1,028

The conserved sequence is transcribed in *Drosophila* and in the mouse, and the transcripts seen in both species hybridize with the same single-stranded probe (see Fig. 2 legend), indicating that a number of different mouse and fly RNAs could code for homologous protein segments. The discovery of these homologies raises the possibility that an unusual protein segment, composed of alternating threonine and glycine residues, and serine and glycine residues, contributes to the organization of biological rhythms in many species. It also focuses attention on this novel protein sequence as encoded by the *Drosophila per* locus. The contribution of this sequence to rhythmic functions in *Drosophila* can be assessed directly by *in vitro* modification followed by transformation of mutant flies.

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obtained when *Drosophila* DNA was used as a probe in these experiments (Fig. 1a), the cloned mouse repeat sequence hybridizes to DNA from the cat and to additional restriction fragments from chicken and man (Fig. 1e). These additional hybridizations may also be due to DNA sequences coding for Thr-Gly and Ser-Gly repeats as the mouse probe is composed chiefly of the conserved DNA sequence, and codons used for threonine, serine and glycine in the mouse cp2.2 clone differ substantially from those used most often to code for these amino acids in the *Drosophila* clone (Fig. 4).

Identification of talin as a major cytoplasmic protein implicated in platelet activation

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During platelet activation there is a major reorganization in the platelet cytoskeleton that accompanies a rapid change in platelet shape^{1,2}. Many of the events associated with activation are attributed to a rise in calcium concentration within the platelet cytoplasm^{3,4}. One direct consequence of the elevated calcium is the activation of a calcium-dependent protease that cleaves a major platelet protein of relative molecular mass (M_r) ~235,000 (235K) to 200K (refs 5, 6). This protein, P235, has been purified⁶ and reported to interact with actin⁷, but the significance of the proteolytic cleavage is unknown. Talin, a cytoskeletal protein in smooth muscle and fibroblasts^{8,9}, binds vinculin¹⁰ and, together with vinculin^{8,9,11,12}, is localized in fibroblasts at sites of actin-membrane attachment. Talin and P235 have similar purification procedures, sedimentation coefficients and Stokes' radii (ref. 6 and Molony *et al.*, unpublished observations). Of particular significance, talin is readily cleaved by proteases from ~215K to a fragment of ~190K²⁷. Given these similarities we have investigated the possible relationship between these proteins. Here we demonstrate that platelet P235 is recognized by anti-talin antibody and that it binds vinculin. Both proteins are cleaved *in vitro* by the calcium-activated protease to yield similar fragments. We conclude that P235 corresponds to the platelet form of talin.

When whole platelets are electrophoresed on SDS-polyacrylamide gels (Fig. 1a), many of the most prominent bands correspond to cytoskeletal components: filamin (actin-binding protein) (250K), myosin (200K), vinculin (130K), α -actinin (100K) and actin (42K). The platelet protein P235 is a major component migrating between filamin and myosin (Fig. 1a, lane 2). When purified chicken gizzard smooth muscle talin is electrophoresed next to the sample of whole platelets (Fig. 1a, lane 3) it appears to migrate to approximately the same position in the gel as P235. However, it is difficult to determine M_r values in this region of a gel with precision, as the thickness of the bands contributes a significant error to the measurements. To compare the apparent M_r s of P235 and talin more accurately, small amounts of the purified proteins as well as those of myosin heavy chain and brain spectrin (fodrin) were examined (Fig. 1b). By this approach, P235 and talin migrate close to each other between the positions of myosin (200K) and the brain spectrin doublet (235K and 240K), but P235 appears to be slightly heavier than talin. The possible identity of P235 with talin was explored using a specific antibody against talin and the Western blot technique (Fig. 2a, b). A crude supernatant from platelets rich in P235, purified P235 and purified talin was electrophoresed on a 10% SDS-polyacrylamide gel (Fig. 2a). The proteins were transferred to a nitrocellulose sheet that was incubated with a rabbit antiserum specific for talin, followed by a second radioiodinated goat antibody directed against the first. The autoradiograph (Fig. 2b) shows that the anti-talin antibody cross-reacts with purified and crude platelet P235 protein.

Previously, several assays have been used to demonstrate that talin binds vinculin^{10,13,14}. If P235 is functionally related to talin it might be expected to interact similarly with vinculin. This possibility was investigated using radioiodinated vinculin to probe P235 in samples transferred from an SDS-polyacrylamide gel to a nitrocellulose sheet. As shown in Fig. 3, P235 and talin are indistinguishable in their ability to bind ¹²⁵I-vinculin specifically.

Fig. 1 a, SDS-polyacrylamide gel electrophoresis of standard proteins (lane 1), whole human platelet proteins (lane 2) and purified smooth muscle talin (lane 3). Platelet P235 (arrow) is a major component of intact platelets. At this concentration, talin migrates close to the position of P235. M_r markers in lane 1 are as follows: myosin, 200K; β -galactosidase, 130K; phosphorylase, 95K; bovine serum albumin (BSA), 68K; ovalbumin, 42K; carbonic anhydrase, 30K. b, Comparison of the electrophoretic mobilities of talin and P235. Proteins were electrophoresed in a 10% SDS-polyacrylamide gel and stained with Coomassie blue. Lane 1, myosin; lane 2, P235; lane 3, talin; lane 4, bovine brain spectrin (fodrin). These light loadings increase resolution, making it possible to compare the mobilities of different proteins more accurately. Note that talin (lane 3) migrates slightly ahead of P235 (lane 2), and that both talin and P235 fall between myosin (200K) and the brain spectrin doublet (235K and 240K).

Methods. SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli²⁰; 10% gels contained 0.13% bis-acrylamide. Outdated human platelet-enriched plasma was used within 1 week of expiration. The platelets were washed extensively according to the method of Collier and Wang⁶, mixed with sample buffer and boiled immediately before electrophoresis. To purify P235, washed platelets were extracted with Triton X-100 as described previously⁶. The material in the supernatant after extraction was enriched in P235 and was used for the subsequent purification of P235 by sequential column chromatography steps on DEAE-cellulose, Sepharose C1-6B and phosphocellulose⁷. Talin was purified from chicken gizzard smooth muscle as described elsewhere⁹ except that chromatography on phosphocellulose was omitted and the final column was hydroxylapatite eluted with a linear gradient of K-phosphate (100–400 mM), pH 7.2. Myosin was from the protein M_r marker kit of Bio-Rad. Bovine brain spectrin was purified by a combination of previously published techniques^{21,22}.

One of the most striking observations concerning platelet P235 is its susceptibility to proteolytic cleavage by the calcium-dependent protease during platelet activation. To determine whether smooth muscle talin is also a substrate for this protease, purified platelet P235 and talin were incubated with purified calcium-dependent protease in the presence of calcium (Fig. 4a). As demonstrated previously⁵, the calcium-dependent protease cleaves platelet P235 to fragments of ~46K and 200K (Fig. 4a, lane 5). Talin also gives rise to a low M_r fragment of ~46K and a single high M_r fragment of 190K (Fig. 4a, lane 4). The difference in M_r s between the larger fragments appears to reflect the difference in size between the intact proteins. Once these two fragments have been generated by the protease, both talin and P235 are refractory to further calcium-dependent proteolysis under the native conditions used here. The addition of leupeptin, a specific inhibitor of the calcium-dependent protease, to the reaction mixture blocks the calcium-dependent cleavage of P235 and talin by the protease (Fig. 4a, lanes 6, 7).

We have also compared the one-dimensional partial peptide maps of P235 and talin using other proteases. With staphylococcal V8 protease (Fig. 4b), both proteins again show an analogous pattern of cleavage to the first proteolytic cut, generating similar-sized fragments of 40K–42K. Note that for both calcium-dependent protease and V8, the major lower M_r fragment generated from talin appears to have a slightly higher M_r than that generated from P235. After prolonged proteolytic digestion with V8 protease, some differences in the pattern of polypeptides generated are seen between the two proteins. Differences are

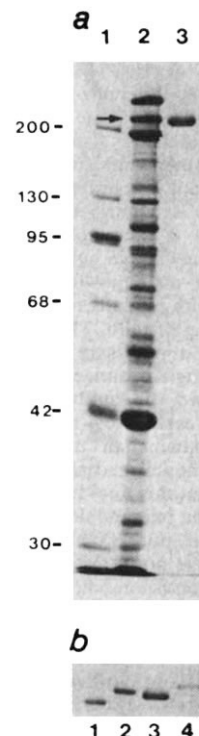
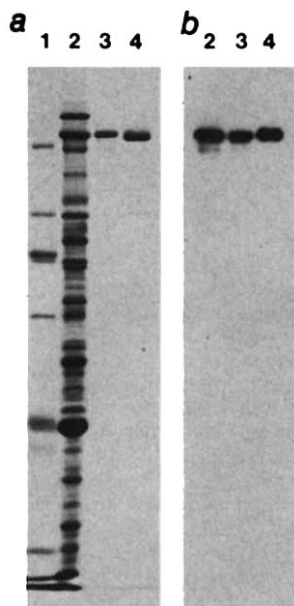


Fig. 2 Immunological cross-reactivity of P235 and talin. *a*, A 10% SDS-polyacrylamide gel of M_r markers as in Fig. 1*a* (lane 1), the P235-enriched Triton X-100 supernatant from platelets (lane 2), purified P235 (lane 3) and purified talin (lane 4). *b*, Immunoblot analysis showing an autoradiograph of an equivalent gel slice with a greatly reduced loading of talin. This reduction was necessary because the original antigen was avian and the antibody is only weakly cross-reactive with mammalian material (our unpublished observations), but this uneven loading for the immunoblot resulted in an apparent shift in the relative positions of the bands corresponding to P235 and talin in the autoradiograph.



Anti-talin antibody specifically binds a protein that co-migrates with P235 in the Triton X-100 extract (lane 2) as well as purified P235 (lane 3) and purified talin (lane 4). The 200K proteolytic fragment of P235 is also visible in lane 2. Affinity purified anti-talin antibody and another antibody raised against an 190K proteolytic fragment of chicken gizzard talin yield the same result (data not shown). Non-immune rabbit serum does not recognize these proteins (data not shown).

Methods. Western blot analysis was carried out by a slight modification of the method of Towbin *et al.*²³. After 60 min incubation in BSA buffer containing 0.05% Tween 20 (ref. 24) and 0.2% gelatin, the nitrocellulose strip was incubated for 90 min with a rabbit polyclonal antibody diluted 1:2 K. The anti-talin antibody was determined to be monospecific by immunoblot and immunoprecipitation experiments (M.C.B., unpublished results). After washing for 30 min, the nitrocellulose strip was incubated for 60 min with $\sim 10^6$ c.p.m. ml^{-1} of affinity-purified goat anti-rabbit IgG radioiodinated by the chloramine-T method²⁵, washed, dried and exposed to X-ray film for autoradiography. All manipulations were carried out at room temperature.

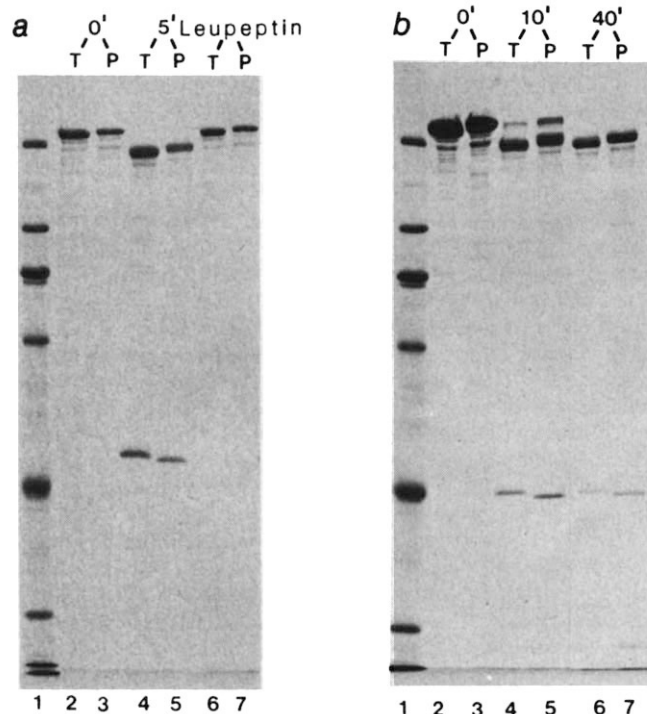


Fig. 3 Platelet P235 binds vinculin. *a*, Photograph of a Coomassie blue-stained SDS-polyacrylamide gel. Lane 1, P235-enriched supernatant obtained after extraction of whole platelets with Triton X-100; lane 2, partially purified human platelet P235; lane 3, purified chicken gizzard talin. *b*, Autoradiograph of an equivalent gel transferred to a nitrocellulose strip and incubated for 90 min with 2×10^4 c.p.m. ml^{-1} ^{125}I -vinculin²⁵. The major vinculin-binding protein in the platelet extract (lane 1) is P235. DEAE-cellulose-purified P235 (lane 2) and talin (lane 3) also bind the labelled vinculin.

Fig. 4 Comparison of the one-dimensional partial proteolytic maps of talin and P235. *a*, Proteolysis by the calcium-dependent protease. Under the conditions described below, the calcium-dependent protease recognizes single sites on both talin and P235 to generate a pair of similar-sized proteolytic fragments for each protein. A 10% SDS-polyacrylamide gel illustrating a typical experiment is shown. Lane 1; M_r marker proteins as described in Fig. 1; lanes 2, 3, talin (lane 2) and P235 (lane 3) before proteolysis; lanes 4, 5, proteolytic fragments that result from talin (lane 4) and P235 (lane 5) after incubation for 5 min at 23 °C with the calcium-dependent protease; lanes 6, 7, talin and P235, respectively, after 30 min at 23 °C in the presence of the calcium-dependent protease plus leupeptin, a specific inhibitor of the protease. The generation of the proteolytic products is completely inhibited by leupeptin even during an extensive incubation period. The protease itself is not visible in the gel at this dilution. *b*, Digestion of talin and P235 with staphylococcal V8 protease. Lane 1, protein standards as in Fig. 1; lanes 2, 3, talin (lane 2) and P235 (lane 3) at 0 min incubation with the protease; lanes 4, 5, talin (lane 4) and P235 (lane 5) after 10 min digestion with V8 protease. Most of the talin and P235 has been cleaved to fragments of 190K and 200K, respectively; also, each has given rise to a low M_r fragment in the range of 40K–42K. By 40 min (lanes 6 and 7) all the talin (lane 6) and P235 (lane 7) has been cleaved to the 190K and 200K products. The lower M_r fragment appears to be reduced relative to the 10-min time point. In some very heavily loaded gels at least four distinct fragments of <40K can be resolved after 30 min incubation with V8 under the conditions described below; at least two of these fragments are shared by both talin and P235 (data not shown).

Methods. *a*, Purified talin and P235 (10.5 μg each) were incubated with the purified calcium-dependent protease at a weight ratio of 1:50 (enzyme: substrate). Proteolysis using the calcium-dependent protease (type II) purified from bovine heart²⁶ was carried out at 23 °C in a total reaction volume of 70 μl containing 35 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol and excess (7 mM) CaCl_2 . The reactions were quenched at various time intervals by the addition of Laemmli sample buffer²⁰ and immediate boiling for 3 min. Addition of leupeptin (1.4 mg ml^{-1}) completely abolished the protease activity. *b*, Purified talin and P235 (16.8 μg each) were incubated at 23 °C with staphylococcal V8 protease at a concentration of 1:100 (enzyme: substrate) in a total volume of 35 μl . The reaction mixture contained 50 mM Tris-HCl, pH 8.0, 3 mM EDTA and 0.1 mM dithiothreitol. The proteolysis was stopped at various time points by boiling for 3 min in Laemmli sample buffer.

also seen when the proteins are digested with other enzymes such as trypsin²⁷ indicating that although the two proteins are related and have similar domains, they are not identical.

Here we demonstrate that P235 and talin are immunologically related. In addition, we show that P235, like talin, binds vinculin, and that both proteins are substrates for the calcium-dependent protease. Previous work has revealed that P235 and talin exhibit similar purification characteristics and biophysical properties. Taken together, these observations lead us to conclude that P235 is the human platelet form of talin.

The identification of P235 as talin brings together two seemingly disparate areas: first, there is a major platelet protein implicated in platelet function; and second, there is a protein localized at sites of actin attachment to the plasma membrane. Platelet P235 seems to be important in some aspects of platelet activation because its rapid and specific proteolytic cleavage correlates with the massive morphological and functional transformation of platelets in response to stimulants such as thrombin^{1,2,5}. The specific role of P235 in platelets is obscure at present because platelet activation is complex and involves several distinct events including an alteration in actin filament content, reorganization of the cytoskeleton, secretion, increased adhesiveness and, finally, aggregation and retraction^{1,2}. It has been estimated that P235 represents between 3 and 8% of total platelet protein⁶, which is considerably higher than the level of talin in fibroblasts. The fact that adhesion and actin-based contractility are specialized functions of platelets may account for the very high levels of talin/P235 in these cells. Alternatively, the abundance of talin/P235 in platelets may indicate other functions of this protein that we had not anticipated from our studies with smooth muscle and fibroblasts.

In fibroblasts, both talin and vinculin are found at sites of tight adhesion to the substrate where actin filaments attach to the plasma membrane. However, despite their provocative location, the role of these proteins in actin-membrane interaction has not been established. Initially, evidence was presented for an interaction of vinculin with actin¹⁵⁻¹⁷, but subsequent work has shown this to be caused by a contaminant in vinculin preparations^{18,19}. P235, on the other hand, has been reported to interact with actin in low-shear viscometric assays⁷. It will be interesting to re-investigate the possibility that smooth muscle talin interacts with actin in light of the demonstration that the two proteins are related by many other criteria.

The significance of the proteolysis of P235 to a 200K fragment that accompanies platelet activation is not clear, but it is intriguing that talin from other sources also exhibits a similar pattern of proteolytic cleavage. We have purified the major proteolytic fragment of talin from smooth muscle and are now comparing the properties of the intact protein with those of the proteolytic fragment. These investigations should provide greater understanding of the function of talin/P235 in both platelets and other cells and may reveal the physiological significance of its cleavage during platelet activation.

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Sequence-induced DNA curvature at the bacteriophage λ origin of replication

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DNA replication in bacteriophage λ begins at a unique origin between residues 39,000 and 39,200 of the λ genome¹. This segment of DNA serves a dual function since it also lies within the coding sequence of the λ replication initiator protein O which binds origin DNA^{2,3}. The λ origin sequence contains four 19-base-pair (bp) segments (iterons) which have dyad symmetry, followed by a 40-bp A+T-rich zone of highly asymmetrical base composition². It was noted earlier⁴ that λ origin DNA exhibits an anomalous electrophoretic mobility on gels; that is, the length of DNA as determined by DNA sequencing is ~20% less than is predicted from electrophoretic mobility. Recent studies of kinetoplast minicircle DNA (K-DNA) from the protozoan *Leishmania tarentolae* have led to the proposal that sequence-induced DNA curvature could account for such electrophoretic anomalies by alteration of the shape of the DNA molecule⁵⁻⁸. We now present evidence that the λ origin contains a static curve.

The λ origin sequence is aligned with K-DNA in Fig. 1, revealing striking correspondence of periodically repeated dA tracts (underlined areas). In K-DNA the centres of the A tracts are separated by an average of 10 bp, that is, approximately one turn of the helix. In the λ sequence the centres of the A tracts show a period of 11 bp within the iteron region, again about one helix turn, but this periodicity degenerates to the right of the iterons in the A+T-rich zone. The best correspondence between K-DNA and λ is in the left half of the origin (iterons IV, III and II). Levene and Crothers, building on ideas of Trifonov and Sussman⁹, have proposed 'wedge' and 'junctional' models of static curvature and applied them successfully to K-DNA. Periodic repetition of runs of A is the main feature leading to static curvature in both these models. An alternative, the purine clash model, is less successful in predicting K-DNA curvature¹⁰.

A hallmark of curved DNA is the anomalously slow electrophoretic mobility of restriction fragments which is more pronounced when the curvature is near the middle of the fragment. To test the λ origin for this property, we used the circular permutation test of Wu and Crothers⁷. We cloned tandem dimers of the ori fragment and cleaved them with a variety of enzymes which cut only once within that sequence, to produce a family of circularly permuted molecules of 1,068 bp and identical base composition (Fig. 2a). These molecules differ in respect to the position of the origin relative to the end of the fragment. Our measurements of the electrophoretic mobility of these fragments are presented in Fig. 2. As plotted in Fig. 2c, the relative size of the fragment reaches a minimum when the *EcoRI* site is near the middle. This site is closest to the λ origin.

To define the region responsible for anomalous mobility more specifically, we cloned a tandem dimer of a shorter fragment of 174 bp containing the λ origin, so that restriction enzymes which

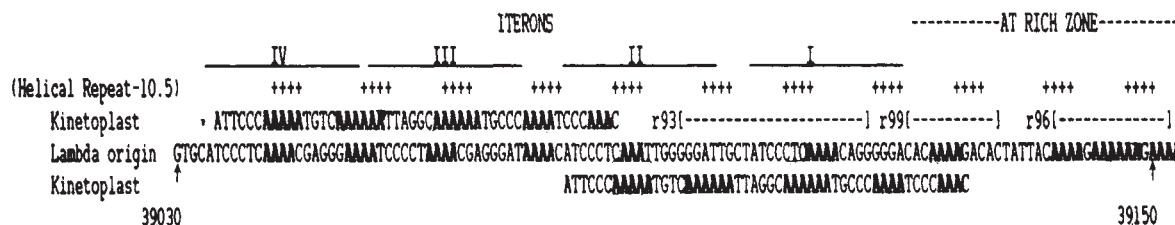


Fig. 1 Sequence comparison of λ iterons and bent DNA from kinetoplast of *Leishmania tarentolae*. The region of kinetoplast DNA shown to be responsible for sequence-dependent curvature⁷ is aligned with λ origin DNA (residues 39,030–39,153)². Since K-DNA is shorter than the λ origin, it is repeated above and below. The λ iterons I to IV, which are known to be the binding sites of λ O protein (ref. 3 and K.Z. and F.R.B., manuscript in preparation), are shown as lines above the sequence. Runs of dA are indicated by underlining. +Symbols alternately spaced 10 and 11 bp apart indicate the 10.5-bp average pitch of DNA in solution. r93, r99 and r96 are deletions of the λ origin sequences. The A + T-rich zone of the λ origin is delineated on the right.

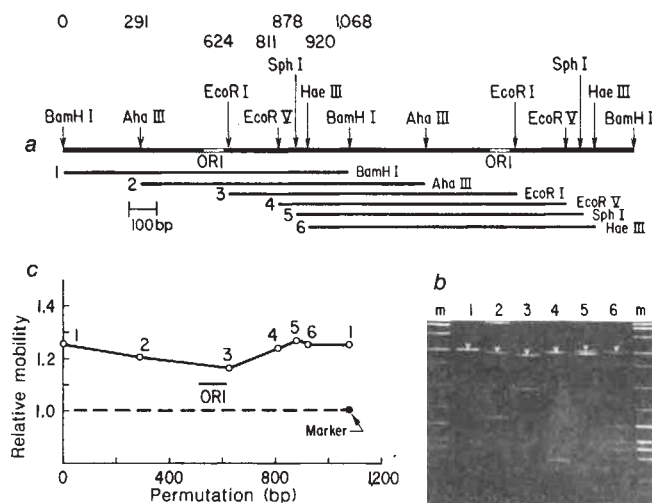


Fig. 2 Permutation analysis of a 1,068-bp fragment containing the λ origin. **a**, A blunt *HincII* fragment isolated from λ DNA was cloned with *Bam*HI linkers as a tandem dimer into the *Bam*HI site of the vector pBR322 (ref. 16). The restriction sites which occur once in each unit of the dimer are indicated and their positions within the fragment are noted above the site. The origin is shown as an open rectangle denoted ORI on the map. Each permuted fragment is shown as a line under the map bounded by its recognition sites and the fragment is numbered on the left and named by the enzyme used to generate it on the right. **b**, Electrophoresis of the permuted fragments in a 7.5% polyacrylamide Tris-borate gel containing 25% glycerol. Plasmid DNA was digested with the enzymes *Bam*HI, *Aha*III, *Eco*RI, *Eco*RV, *Sph*I and *Hae*III. Lanes are indicated according to the numbers in **a**. White arrows indicate the permuted fragments derived from each restriction digest which correspond to the numbered fragments in **a**. Lanes m are markers (*Hae*III plus *Bst*NI digest of pBR322) used to size the fragments. **c**, Relative sizes of the permuted fragments in **b** are plotted against the positions of the restriction site (permutation) in one unit of the tandem dimer. Relative size is determined as the apparent size of the fragment divided by its actual size. The position of the origin in the permuted fragment is denoted by ORI. The dotted line describes the mobility of a sequenced *Bst*NI marker band of 1,068 bp from pBR322 which migrates normally.

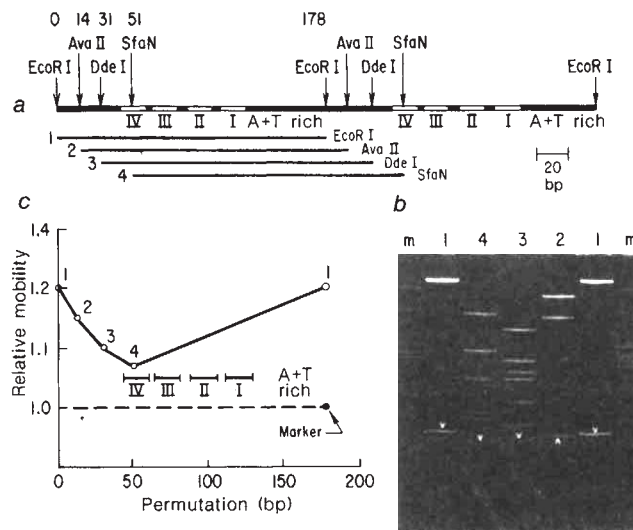


Fig. 3 Permutation analysis of the 178-bp subfragment containing the λ origin. **a**, A 174-bp *Fnu*HI/*Eco*RI fragment containing the λ origin was filled-in with DNA polymerase I (Klenow fragment) and *Eco*RI linkers were ligated to it for cloning as a tandem dimer into the *Eco*RI site of the vector pUC18 (ref. 17). See Fig. 1 for details. The iterons are shown as open rectangles denoted IV, III, II, I, according to their position in the λ sequence. 'A + T rich' indicates the position of the A + T stretch in the origin. **b**, Electrophoresis of permuted fragments in a 7.5% polyacrylamide, Tris-borate gel containing 25% glycerol. Plasmid DNA was digested with the enzymes *Eco*RI, *Ava*III, *Dde*I and *Sfa*NI. **c**, Relative sizes of the permuted fragments in **b** are plotted against the position of the restriction site (permutation) in one unit of the tandem dimer. The dotted line describes the mobility of a sequenced marker band of 178 bp which migrates normally.

cut closer to the putative curve could be investigated (Fig. 3a). The result is plotted in Fig. 3c. The minimum point in this case is the *Sfa*NI site, which resides within iteron IV, suggesting that this is the region of maximum curvature. It is also the region of closest similarity to K-DNA.

Several deletion mutants of λ are available which might be used to define more precisely which portion of the λ origin contains the curve. The r99 and r96 mutations are deletions of 12 and 15 bp, respectively, which remove portions of the A + T-rich zone at the right end of the origin. The r93 mutation is a 24-bp deletion in the iteron region (see Fig. 1). Each of these

mutations was cloned into a plasmid¹¹. Electrophoresis of *Bgl*II/*Eco*RI fragments from the mutants is presented in Fig. 4. The degree of anomalous mobility is expressed as the ratio of true size to apparent size (relative size). On this scale the full curving effect corresponds to a factor of 1.17 with normal uncurved DNA as 1.00. The A + T-rich zone mutants r99 and r96 had no effect on anomalous migration; their ratios were 1.18. Thus, the anomaly is not due to runs of A residues *per se*; their periodic spacing seems important.

On the other hand, the relative size anomaly observed for the *Bgl*II/*Eco*RI fragment from r93 is 1.13, indicating that about one-quarter of the anomaly disappears whereas three-quarters is retained. This makes sense since Ori_{r93} has three-quarters of the normal iteron number. It would be interesting to examine whether more extensive deletions of the origin would have proportionate effects.

The magnitude of the relative size anomaly shown by λ origin DNA is small compared with that of K-DNA measured in a

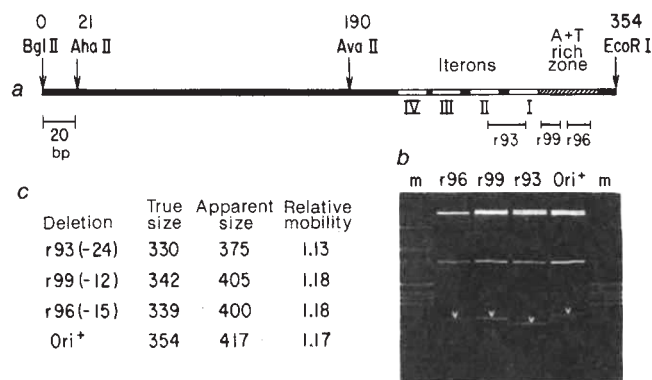


Fig. 4 Mobility analysis of restriction fragments containing deletions within the origin. **a**, The *Bgl*II/*Eco*RI fragments from the plasmids pKdT3 (Ori⁺), pKdT93 (Ori⁻ r93), pKdT99 (Ori⁻ r99), and pKdT96 (Ori⁻ r96) were studied¹¹. The map indicates the positions of each of the *r* mutant deletions within the wild-type fragment. The iterons (open rectangles), A+T-rich zone (hatched rectangle) and a few relevant restriction sites are shown. **b**, Electrophoresis of *Bgl*II/*Eco*RI digests of the *r* mutant plasmid DNAs. Digests were electrophoresed on 7.5% acrylamide, Tris-borate gels containing 25% glycerol. **c**, Characterization of the *Bgl*II/*Eco*RI fragments of the Ori⁻ mutants. The size of the deletion (in base pairs) is noted in parentheses next to each mutant. True size from DNA sequencing experiments¹¹ is listed alongside as well as the apparent size from measurement of the gel in **b**.

similar gel system. Greater than twofold differences between apparent and real size can occur in the case of K-DNA^{7,8}. This difference may be caused by differences in the size of the curve dictated by sequence differences. The longer helical repeat (11 bp) in the iteron region may also contribute to the smaller mobility effects seen with λ origin DNA.

Computer analysis of helix trajectories using the computer model of Levene and Crothers¹⁰ and assuming a 5° tilt for each A dinucleotide, predicts a bend of about 35° for Ori and 87° for K-DNA. These predictions are based on an unconfirmed model, but they are at least consistent with the idea that the magnitudes of the mobility effects may depend on the size of the curve.

Gough and Lilley¹² recently demonstrated that mobility effects of the type described here can be generated by pseudo-cruciform junctions in DNA rather than by curving of double-stranded DNA. Although cloverleaf structures have been described as a possible conformation for the λ iterons¹³, no single-stranded character has been detected in this region by analysis with single-strand-specific S₁ nuclease digestion (data not shown) or DNase I (ref. 3). In light of this and the extraordinary similarity with K-DNA, we conclude that λ origin DNA in solution is bi-helical and, like K-DNA, stably curved.

We find it remarkable that the λ origin sequence embodies three distinct functions in the same piece of DNA: (1) the O protein-coding region; (2) the sites to which O protein binds; and (3) a site of DNA curvature.

The dA stretches at the centre of iteron symmetry probably generate wedges leading to curvature, whereas the G+C-rich portions in between, which are highly conserved amongst λ iterons, are probably involved in contacts with the O protein (K.Z., S. H. Wickner and F.R.B., manuscript in preparation). The regions between dA tracts show no sequence pattern in K-DNA⁵.

Our finding represents the first example of a site-specific DNA binding protein whose binding site is within statically curved DNA. Cyclic AMP receptor protein is reported to induce bending in the *lac* control region on binding to it⁷ and this also could be a feature of the O⁻ ori interaction. Echols *et al.*¹⁴ have reported a considerable shortening of DNA fragments containing the λ origin on binding to O protein. They suggest the ori DNA folds within the protein complex. Curving of λ origin DNA may be

critical to its ability to wrap or fold in binding O, and thus be directly related to initiation of DNA replication.

We thank D. Moore who noticed the sequence similarity between K-DNA and the λ origin and J. Schroeder for programming the Levene model on our IBM personal computer. We thank D. L. Daniels for help with the manuscript, A. Hopfensperger for art work and N. Peterson for secretarial assistance. This is paper 2794 from the Laboratory of Genetics, University of Wisconsin, and is supported by NIH grants GM 21812 to F.R.B. and NIH GM 07133 to the Department of Genetics.

Note added in proof: Trifinov recently estimated the A-A wedge angle as 8.6° based on ring closure experiments with a defined synthetic oligonucleotide substrate¹⁵. With this assumption the computer predicts a 90° angle for the λ ori.

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Regulatory elements controlling chorion gene expression are conserved between flies and moths

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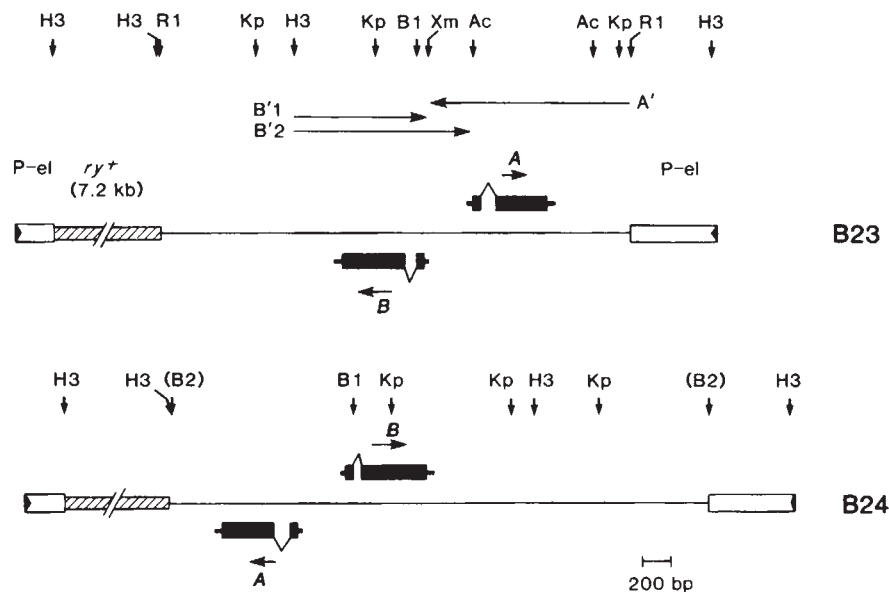
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Flies and moths are approximately as distant phylogenetically as are mammals and birds. In terms of morphology, physiology and biochemistry, the complex proteinaceous eggshell or chorion differs substantially in these two insect groups, which are typified by *Drosophila melanogaster* and *Bombyx mori*. The major chorion proteins of moths are encoded by two families of genes, *A* and *B*, which have no obvious homologues in flies¹. Unlike *Drosophila*, where chorion genes are oriented in tandem, moths show mostly chorion gene pairs (*A* plus *B*) that are divergently transcribed and coordinately expressed. The 5' ends of the paired genes are separated by a DNA segment of only 300±50 base pairs, which may well include at least some of the *cis*-regulatory elements necessary for gene expression. Despite these differences, we have tested whether moth chorion genes might be expressed in flies. Cloned DNA fragments bearing moth chorion genes were introduced into the *Drosophila* germ line by P-element-mediated transformation^{2,3}. Analysis of RNAs from transformed lines revealed that the genes are expressed with correct sex, tissue and temporal specificity, resulting in the accumulation of abundant moth chorion transcripts in late fly follicles.

Two cloned genomic fragments (3.1 and 3.8 kilobases (kb)) from the chorion locus of *B. mori* were selected for *in vivo* studies of regulatory elements. Each fragment contains a single *A/B* gene pair and immediately flanking sequences. The 3.1-kb fragment has been fully sequenced (N. Spoerel, personal com-

Fig. 1 Structure of transformation vectors containing *B. mori* chorion genes. Open boxes, P-element sequences containing the terminal inverted repeats (black triangles). Striped box, *ry*⁺ sequences encompassing the XDH structural gene. Thin line, *B. mori* genomic sequences. The exons of the divergently transcribed chorion genes are indicated by filled boxes, wide for coding sequences and narrow for untranslated regions; connecting lines represent the single intron of each gene. Arrows accompanying the A or B gene designation indicate the direction of transcription. Other arrows indicate the orientation and size of SP6-generated cRNA probes¹³ used for Northern and S₁ protection analyses. Restriction endonuclease sites pertinent to this study are indicated. Ac, *AccI*; B1, *BglI*; B2, *BglII*; H3, *HindIII*; Kp, *KpnI*; R1, *EcoRI*; Xm, *XmnI*. **Methods.** Plasmid pB23 contains a completely sequenced 3.1-kb *B. mori* *EcoRI* genomic fragment, encompassing the A/B chorion gene pair known as A.L12/B.L12 and immediate flanking sequences (N. Spoerel, personal communication). The fragment was inserted into the *EcoRI* site of the polylinker of the pCar20 *Drosophila* transformation vector⁴. Plasmid pB24 contains a 3.8-kb *BglII* fragment inserted into the unique *HpaI* site of the same vector. This fragment contains the highly homologous (but not identical) A.L9/B.L9 chorion gene pair. The vector segments integrated into *Drosophila* chromosomes to generate lines of the B23 or B24 series are presented above. *Drosophila* transformation was carried out essentially as described^{1,2}. Embryos of the *cn*; *ry*⁴² strain were injected with a DNA mixture of 0.5 mg ml⁻¹ vector and 0.25 mg ml⁻¹ helper plasmid p25.7WC (G. Rubin, personal communication). Resulting F₀ adults were mated with the parental strain and individual *ry*⁺ transformants in the F₁ progeny were used to establish lines. The transformation efficiency was 1–5% of the injected embryos. Genomic DNA isolated from adult flies was restricted with appropriate enzymes for Southern analysis¹⁴ using *ry*⁺-specific and/or chorion-specific probes. Lines containing single inserts and unique line-specific junction fragments were selected.



munication). These fragments were inserted individually, in opposite orientations, in the transformation vector Carnegie-20 (Fig. 1). The vector bears the essential terminal portions of the transposable P-element, flanking a polylinker that provides convenient cloning sites and the *ry*⁺ structural gene for xanthine dehydrogenase, which provides a visible marker for transformation⁴.

The constructs were injected into *cn*; *ry* *Drosophila* embryos, and *ry*⁺ transformant lines were selected. Southern blot analyses of genomic DNA confirmed that these lines also carried the moth genes, as expected. Six independent lines (series B23) were recovered from the 3.1-kb construct and four independent lines (series B24) from the 3.8-kb construct. Independence of the lines was confirmed by detecting in Southern blots variable line-specific junction fragments, spanning part of the insert and the chromosomal insertion site. Figure 2 shows typical results of RNA blot analyses from three such lines, representing both gene pairs and orientations. As assayed with the single-stranded probes indicated in Fig. 1, the A- and B-specific transcripts are absent from the parental strain and from transformed males or ovariectomized females, but are prominent in total ovarian RNAs from all transformed fly lines. Clearly, the transduced genes are transcribed with correct sex and organ specificity. These specific transcripts are polyadenylated, very abundant and comparable in size to the authentic moth chorion RNAs (although the A-specific transcripts exhibit size heterogeneity, the reason for which is discussed below).

Specific transcripts are present in all 10 transformed lines, and are more abundant in the B23 series. Position effects on expression are relatively minor (Fig. 2): independent lines bearing the same construct show only a threefold variation in transcript abundance, except for a homozygous lethal transformant in which the transcript abundance is 15% of the maximum. Unlike the endogenous fly chorion genes, the moth genes are not amplified in the ovaries of transformants, as they are not in the moths themselves (data not shown). In the most abundantly expressing lines, the transcripts reach a steady-state level corresponding to 2.5% of the *Drosophila s15-1* (A1) chorion messenger RNA. As the latter is encoded by a locus that amplifies approximately 80-fold in the fly follicles⁵, the abundance of moth transcripts per gene copy is up to double the abundance of a major fly chorion mRNA.

To examine in more detail the developmental specificity of

transcription, RNAs from staged follicles were analysed. Figure 3 shows that the moth chorion transcripts are expressed mostly in the late period of choriogenesis. Furthermore, the transcripts are also absent from newly laid eggs (results not shown), indicating by subtraction that, like mRNAs of endogenous chorion genes, they are produced in the follicular epithelial cells which are shed at ovulation. Thus, the transcripts show marked tissue and temporal specificity, as well as organ and sex restriction. The paired A and B genes are transcribed completely in parallel, in agreement with their coordinate expression in moths.

Because choriogenesis is so different in moths and flies, it is not possible to equate specific choriogenic stages in the two groups. However, it is intriguing that the moth genes show a bimodal profile of activation. Transcripts are present at a relatively low level in the early stages of oogenesis, then decline substantially in abundance and finally reappear at high levels towards the end of choriogenesis. This behaviour is reminiscent of and closely follows the transcriptional profile of the 'late' *Drosophila s15-1* chorion gene, which produces a transient, non-polysome-associated transcript before choriogenesis^{6,7}. The ratio of premature to late-stage transcripts is even higher for the moth genes than for *s15-1*, but this difference can be accounted for by the late amplification of *s15-1* and the absence of moth gene amplification.

To characterize the moth chorion transcripts more fully, their 5' ends and splicing patterns were examined by S₁ nuclease protection analysis, using uniformly labelled complementary RNA probes (Fig. 4). Like all other chorion genes studied to date, the sequenced A and B genes of the B23 series construct each have two exons, of which the minor one is at the 5' end (N. Spoerel, personal communication; see also Fig. 1). RNA from transformed flies or moth ovaries protects two B cRNA fragments, corresponding both to the sizes expected from the sequence and to major bands observed in parallel experiments with authentic moth ovarian RNA. Thus, the moth chorion B gene mRNA appears to be properly initiated and spliced in the *Drosophila* follicular cells. Similar analysis of the A gene transcripts indicates that they also are properly initiated and spliced, but that multiple polyadenylation sites are used in *Drosophila*, one being the same as that used by *Bombyx* and the rest occurring within 120 bp downstream of this site (data not shown). This results in the size heterogeneity evident in Northern analyses (Figs 2, 3).

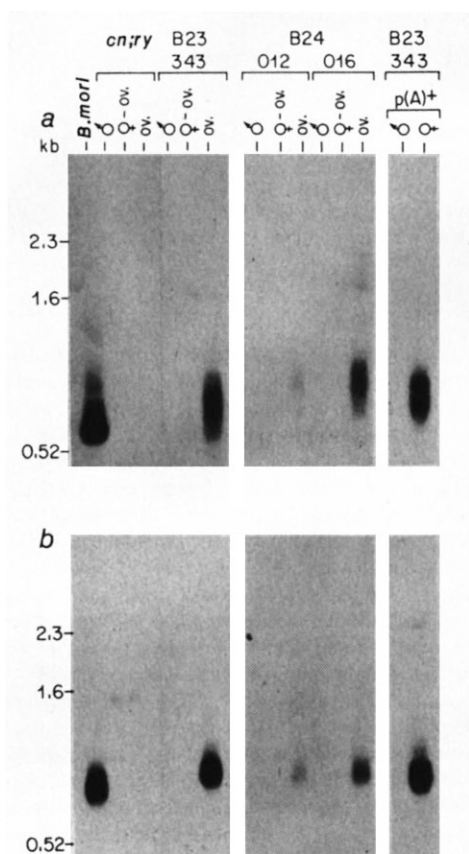


Fig. 2 Sex and organ specificity of silkworm chorion gene transcripts. Total RNA preparations were glyoxylated¹⁵, fractionated by electrophoresis, blotted onto nylon membrane filters and hybridized with A gene-specific (a) or B gene-specific (b) probes (A' and B' in Fig. 1). *B. mori*, 0.1 µg total ovarian RNA (under the conditions of hybridization, transcripts from most members of a chorion gene family are detected). *cn*; *ry*, Parental strain *Drosophila*. B23-343, transformant line representing the B23 series. B24-012 and B24-016, transformant lines representing the B24 series. Each lane contained 10 µg total RNA isolated from males (♂), from females from which the ovaries had been removed by dissection (♀-ov.) or from ovaries (ov.). (A)⁺, 5 µg oligo(dT)-selected mRNA isolated from male or female transformed *Drosophila*. Note the prominent female- and ovary-specific transcripts, which are absent from the parental line. The migration and size (in kilobases) of glyoxylated DNA markers are indicated on the left.

Methods. RNA was isolated using a modification of the urea/SDS method. Briefly, tissues were homogenized in a 1:1 mixture of phenol and 8 M urea, 1% SDS, 0.3 M LiCl, 0.1 M Tris-HCl pH 8.1, 10 mM EDTA and 0.1 M β-mercaptoethanol, two volumes of chloroform was added, and the resulting aqueous phase was extracted twice with phenol/chloroform/isoamyl alcohol (20:20:1) and twice with chloroform. Following ethanol precipitation in the presence of 2 M ammonium acetate, the nucleic acids were dissolved in H₂O and RNA was precipitated by 2.5 M LiCl in the presence of 4 M urea at -20 °C for 8-18 h. Glyoxylated RNAs were fractionated on 1.8% agarose gels and stained by immersion in 50 mM NaOH, 0.5 µg ml⁻¹ ethidium bromide, followed by neutralization in 0.1 M Tris-HCl, pH 7.0; 10×SSC, 0.5 µg ml⁻¹ ethidium bromide, in order to verify the uniformity of RNA amounts loaded in each lane. Gels were blotted on Genescreen (NEN) with 10×SSC and the RNA was crosslinked to the filter by ultraviolet irradiation. SP6-derived cRNA probes specific for A or B genes were prepared¹³ and reduced in average size to 200 bases before use, by controlled alkaline hydrolysis¹⁶. Filters were hybridized with 1-3 × 10⁶ c.p.m. ml⁻¹ of probe in 0.1 M NaHPO₄, 0.05 M Na₂P₂O₇, 10 mM EDTA, 7% SDS, 0.5 mg ml⁻¹ sheared herring sperm DNA, at 78 °C for 36 h, washed¹⁷ and exposed to Kodak XAR-5 film using Cronex Intensifier Screens (DuPont). A faint B-cross-hybridizing endogenous transcript of ~1.6 kb was sometimes seen in non-ovarian tissues of non-transformed *Drosophila*; this cross-hybridization is due to the relatively low stringency of cRNA probes at 78 °C, is greatly reduced if hybridization is carried out at 83 °C, and is not detectable by nick-translated DNA probes at 68 °C. Very minor amounts of high-relative molecular mass, nonspecific transcripts of the moth genes were also seen in some long autoradiographic exposures of both male and female RNA; according to S₁ analyses these are readthrough transcripts from external promoters (data not shown).

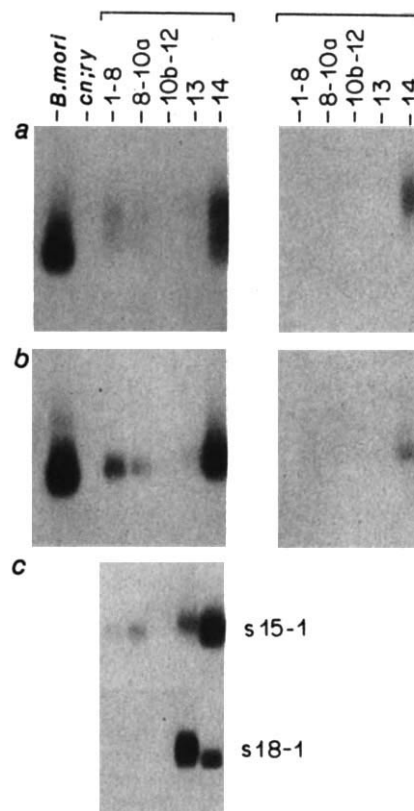


Fig. 3 Temporal specificity of silkworm chorion gene expression. a, A gene-specific probe (A' in Fig. 1). b, B gene-specific probe (B' in Fig. 1). c, Probes specific for *Drosophila* chorion gene *s15-1* or *s18-1* (refs 6, 7). Note that the moth A and B genes are coordinately expressed, with a bimodal temporal pattern resembling that of the endogenous *s15-1* chorion gene. Follicles representing the various stages (1-14) of *Drosophila* oogenesis¹⁸ were isolated from ovaries of female B23-343 or B24-012 transformant flies. Stage-specific RNAs were prepared and analysed as described in Fig. 2 legend. *B. mori*, 0.05 µg total ovarian *B. mori* RNA. *cn*; *ry*, 15 µg total ovarian RNA from parental strain *Drosophila*. Lanes labelled 1-8 to 14 contained 15 µg total nucleic acid isolated from follicles at the corresponding stages of development.

Although the time since the last common ancestor of flies and moths is not known accurately, 240 million years is probably a minimum estimate. This time of separation is comparable to that between birds and mammals. Unambiguous Diptera existed in the Lower Jurassic (some 180 million years ago) and what are thought to be ancient dipterous forms are known from the Permian (240 million years ago)⁸. The Lepidoptera are rather recently evolved insects, with their earliest forms known from the Lower Cretaceous (130 million years ago), but they are closely related to the much more ancient Trichoptera (caddis flies). Both Lepidoptera and Trichoptera apparently evolved from one branch of primitive scorpion flies, while the Diptera and their relatives evolved from another branch^{9,10}. These two branches must have separated before the Upper Permian (240 million years ago), since by that time unambiguously recognizable Trichoptera as well as ancient dipterous forms were already extant^{8,10}.

Although some regulatory elements involved in the physiological heat-shock response are recognizable between flies and mammals¹¹, and expression of the chicken transferrin gene is enhanced moderately in the liver of transgenic mice¹², to our knowledge this is the first report of such prolonged evolutionary conservation of elements that confer clearcut developmental regulation (sex, tissue and temporal specificity of gene expression). Both *trans*- and *cis*-acting regulatory elements responsible for developmentally specific chorion gene expression must have been conserved: for expression in our

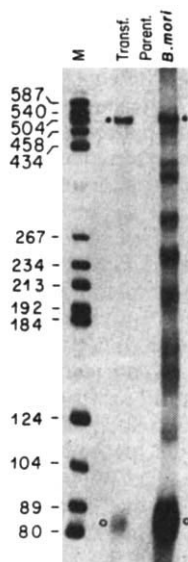


Fig. 4 S_1 nuclease protection analysis of silkmoth chorion gene transcripts. The analysis used single-stranded cRNA probes (B'2 in Fig. 1), uniformly labelled with [α - 32 P]GTP¹³. Hybridizations were carried out at 50 °C in 80% HCONH₂, 0.4 M NaCl, 40 mM PIPES, pH 6.8, 10 mM EDTA for 18 h. Unhybridized RNA was subsequently digested with 2,000 units of S_1 nuclease¹⁹ at 37 °C for 1 h. Protected fragments were glyoxylated and electrophoresed in a 5% polyacrylamide gel in 10 mM sodium phosphate buffer, pH 6.8. M, an end-labelled and glyoxylated *Hae*III digest of pBR322 DNA, providing size markers of indicated lengths (in nucleotides). Transf., fragments protected by 10 μ g ovarian RNA from transformant *Drosophila* line B23-343; Parent., absence of protection by 10 μ g ovarian RNA from the parental strain. *B. mori*, fragments protected by 1 μ g silkmoth ovarian RNA. Protected fragments corresponding to the small and large exon are indicated by ○ and ●, respectively. Other bands in the *B. mori* lane are due to partial protection by homologous but not identical transcripts of other members of the *B* gene family, as well as to secondary structure of the probe.

transformants, *trans* regulators of fly origin must have interacted with moth *cis* regulators contained within the transduced DNA fragments. Indeed, the necessity of interaction between *cis* and *trans* elements if either one is to function may account for their remarkable evolutionary conservation. Further experiments, including surrogate genetics, should help to identify these regulatory elements and lead to an understanding of their interaction.

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Sequence of the precursor of the chloroplast thylakoid lumen protein plastocyanin

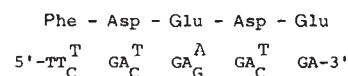
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In photosynthetic eukaryotes most chloroplast proteins are encoded by nuclear DNA^{1,2}. These proteins are synthesized as precursor proteins that are post-translationally taken up by chloroplasts and directed towards their functional compartment²⁻⁴. One of these proteins, plastocyanin, is a photosynthetic electron carrier that is functional in the thylakoid lumen⁵ and therefore probably has to cross three membranes to arrive at its final location⁶. We have isolated a plastocyanin-specific complementary DNA clone from a *Silene pratensis* (white campion) cDNA library using a mixed oligonucleotide hybridization probe and hybrid-released translation. The isolated clone contained the nucleotide sequence for the mature protein and the complete transit peptide, which consists of a hydrophobic, 66-residue-long stretch of amino acids interspersed with positively charged residues. This precursor protein is most probably processed between two alanine residues. Comparison of the transit peptide of the plastocyanin precursor protein (in the thylakoid lumen) with those of the precursor proteins for ferredoxin and the small subunit of ribulose biphosphate carboxylase (in the stroma) and with that of the chlorophyll *a/b* binding protein (inserted in the thylakoid membrane) revealed no similarities, except for a region of 14 residues at the amino-terminal end.

The unique chloroplast structure divides this organelle in at least six different compartments: outer and inner membrane, intermembrane space, stroma, thylakoid and thylakoid lumen. Therefore, chloroplast precursor proteins must contain information for both chloroplast specificity and routing of the mature proteins towards their specific compartments. Concomitant with this process the precursor proteins are proteolytically cleaved to their mature size²⁻⁴. It has been shown^{7,8} that the transit peptide of the chloroplast stroma located small subunit of ribulose biphosphate carboxylase can direct a foreign protein into the chloroplast stroma both *in vivo* and *in vitro*. For the analysis of the mechanism of chloroplast protein uptake and distribution, information about proteins that are directed towards different chloroplast compartments is necessary. Therefore, we cloned and analysed the nucleotide sequence encoding the precursor of plastocyanin. The special transport properties of the plastocyanin precursor protein may be reflected by the exceptional size of its transit peptide, which for pea was reported to have a relative molecular mass (M_r) of ~15,000 (ref. 9). Recently, however, M_r values of 7,000 and 8,000 were reported for the wheat and barley plastocyanin transit peptide, respectively¹⁰.

Plastocyanin-specific cDNA clones were isolated from a *S. pratensis* cDNA library. For this purpose a synthetic oligonucleotide mixture (16 × 14-mers), corresponding to a conserved part of the protein, was synthesized comprising amino acids 41-45 (ref. 11)



and used to screen a cDNA library¹². Fifteen positive clones were isolated, of which only one selected for plastocyanin-specific messenger RNAs in a hybrid released translation and immunoprecipitation experiment (Fig. 1). This clone, pPC1, contained a cDNA insert of ~500 nucleotides and was shown

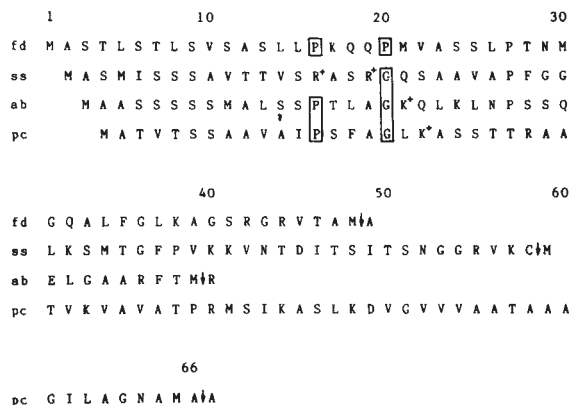


Fig. 4 Comparison of the published transit sequences of chloroplast precursor proteins aligned at the amino-terminal end. The beginning of the Pro- and Gly-containing region with the first basic amino acids is indicated. fd, *S. pratensis* ferredoxin¹² (stroma); ss, pea small subunit¹³ (stroma); ab, pea chlorophyll *a/b* binding protein¹³ (thylakoid membrane); pc, *S. pratensis* plastocyanin (thylakoid lumen). Processing sites are indicated (arrows). The presumed processing site is shown^{13,21} for the chlorophyll *a/b* binding protein.

The transit peptide sequences of three other chloroplast-imported proteins have been determined^{12,13}. Comparison of the amino-acid sequence of all four transit peptides, aligned by using the processing site as a reference point, does not reveal any apparent homology, nor do their hydrophilicity plots (data not shown). However, if the initiator methionine is used as a reference point, the four sequences all begin with a stretch of ~14 uncharged amino acids in which hydroxy-amino acids (Ser and Thr) are abundant (Fig. 4). This region is followed by a Pro- and Gly-containing region ~6 amino acids in length with the first residue(s) strongly basic, after which point there is no further similarity. At the processing sites of the four precursor sequences there is no consensus amino-acid sequence. For three of the transit sequences, strongly basic amino acids are present ~4 residues in front of the processing site, but for plastocyanin no such amino acid is found. Instead the plastocyanin processing site is preceded by a Val- and Ala-rich region, 20 amino acids in length. These amino acids can form a membrane-spanning region and, because this region is unique to the plastocyanin precursor, could be involved in movement across the thylakoid membrane. Methionine residues are present at or near the processing site in all four precursors. The lack of homology, especially near the processing site, seems to contrast with the observation that proteases are probably not precursor-specific¹⁰.

With the isolation of a plastocyanin-specific cDNA, clones for precursor proteins directed towards three different chloroplast locations are now available. These clones will be used to study the mechanism by which chloroplasts import proteins and distribute them towards the various organellar compartments.

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DNA sequence at the end of IS1 required for transposition

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The insertion sequence IS1 belongs to a class of bacterial transposable genetic elements that can form compound transposons in which two copies of IS1 flank an otherwise non-transposable segment of DNA. IS1 differs from other known elements of this class (such as IS10, IS50 and IS903) in several respects. It is one of the smallest known insertion elements¹⁻³, exhibits a relatively complex array of open reading frames³, is present in the chromosomes of various Enterobacteria, in some cases in many copies^{4,5}, and its insertion can result in the duplication of either 8 or 9 base pairs (bp) in the target DNA³. Furthermore, although, like other members of the compound class, it seems to undergo direct transposition, IS1 also promotes replicon fusion (co-integrate formation) at a relatively high frequency⁶. Like all other elements studied to date, the integrity of the extremities of IS1 are essential for efficient transposition⁷. We have constructed a test system to determine the minimal DNA sequences at the extremities of IS1 required for transposition. Sequential deletions of the end sequences reveal that 21-25 bp of an isolated extremity are sufficient for transposition. A specific sequence 13-23 bp from the ends, defining the edge of the minimal sequence, is implicated as an essential site. The sites, symmetrically arrayed at both ends of IS1, correspond to the apparent consensus sequence of the known binding sites for the *Escherichia coli* DNA-binding protein (called integration host factor or IHF) which is required for the site-specific recombination that leads to integration of bacteriophage λ into the bacterial genome^{8,9,26}. The sites at the ends of IS1 may thus bind a host protein, such as JHF or a related protein, that is involved in regulating the transposition apparatus.

As a first step in defining the signal sequences carried by the extremities of IS1, we have determined the minimum terminal sequence required for transposition. For this, we applied the method used previously for IS50, IS10 and bacteriophage Mu, that is, monitoring the transposition activity of compound transposons in which one or both flanking elements have been truncated to varying degrees by the exonuclease Bal31 (refs 10-13).

Figure 1 shows the construction of the transposons used. They are composed of an IS1-flanked spectinomycin (Sp)/streptomycin (Sm) resistance determinant. The efficiency of transposition of Sp^R/Sm^R from these IS1-flanked transposons was determined using a 'mating-out' assay (see Table 1 legend)⁶. Table 1 shows our results. The frequencies of transposition of Sp^R/Sm^R from the plasmids pOC15 and pOC17 are comparable (as is that of co-integrate formation), indicating that the 76-bp extremity of IS1 carried by pOC15 is as efficient in promoting wild-type

levels of transposition as an intact copy of the element carried by pOC17. The slightly higher level of transposition found in truncated IS1 (pOC15) is reproducible, although we cannot explain this.

Our results also indicate that functional ends of IS1 must be present in the correct orientation for transposition to occur. Thus, 'inverse transposition' of ampicillin/carbenicillin resistance determinants occurs at a frequency of 4.3×10^{-7} from plasmid pOC17 but is undetectable at $<1 \times 10^{-8}$ from pOC15.

To determine the minimum sequence at the extremity of IS1 required for transposition, we constructed a series of plasmids carrying *Bal*31-generated deletions into the 76-bp IS1 extremity (Fig. 1 legend). The size of the *Bal*31 deletions was determined to within ~5 bp by analysis with suitable restriction endonucleases. The transposition activity of 20 plasmids (Fig. 2a) indicates that a threshold size of 20–25 bp is necessary for efficient transposition.

To define the minimum sequence more precisely, the region of IS1 remaining in six representative plasmids was determined using the Maxam and Gilbert sequencing method¹⁴ (Fig. 2b, Table 1). Plasmids pOC22.235, pOC22.212 and pOC22.222 (76, 28 and 25 bp of the IS1 end, respectively) allow transposition to occur at frequencies similar to those exhibited by pOC15 and pOC17. Plasmid pOC22.228 (21 bp) shows a 10-fold reduction whereas pOC22.236 (20 bp) and pOC22.218 (16 bp) show undetectable levels of transposition ($<0.5\%$ of wild type).

Table 1 Transposition activity of IS1-flanked transposons

Plasmid	No. of base pairs remaining	Co-integrate frequency ($\times 10^{-6}$)	Transposition frequency ($\times 10^{-6}$)	
			Ap(Cb)	Sp/Sm
pOC17	768	4.9	0.43	0.74
pOC15	76	1.2	<0.01	2.0
pOC22.235	76	1.9	NT	4.4
pOC22.212	28	5.8	NT	5.5
pOC22.222	25	6.8	NT	5.0
pOC22.228	21	2.4	NT	0.57
pOC22.236	20	1.2	NT	<0.01
pOC22.218	16	1.8	NT	<0.01

Strain LC1042 (*Leu-Trp-MetA-Ile-ArgG-ProcC-Thi-Ara-Lac-Xyl-Mtl-Gal Str^R T6^R recA/pOX38Km*) carrying each of the donor plasmids was crossed with LC799 (*Thy-Leu-Lac-Thi-Nal^R P1^R A^R*) and ex-conjugants resistant to kanamycin (Km^R), spectinomycin (Sp^R) or ampicillin/carbenicillin (Cb^R) were selected on L agar plates²² supplemented with the appropriate antibiotics. Mating conditions have been described previously^{6,23}. To distinguish co-integrates between the donor and recipient replicons from direct transposition, Sp^R/Sm^R clones were analysed for Cb^R and Cb^R clones for Sp^R/Sm^R . Sp^R/Sm^R , Cb^R ex-conjugants were scored as co-integrates, Sp^R/Sm^R , Cb^S clones and Cb^R , Sp^S/Sm^S clones were scored as direct transposition of the respective IS1-flanked regions of the donor replicon. Frequencies are expressed as a fraction of the transfer of pOX38Km alone. No correction for the increased mating efficiency of pOX38 plasmids which carry the origin of pBR322, that is, all Cb^R ex-conjugants⁶, has been made in the above results. Drug concentrations used were: Km , $25 \mu\text{g ml}^{-1}$; Nal , $35 \mu\text{g ml}^{-1}$; Sp , $30 \mu\text{g ml}^{-1}$; Sm , $10 \mu\text{g ml}^{-1}$; Cb , $500 \mu\text{g ml}^{-1}$. NT, not tested.

Thus, 25 bp at one end of IS1 is sufficient to promote normal levels of transposition in conjunction with a second, intact copy of the element. The extremities of IS1 carry relatively large inverted sequences, IRL and IRR, which show extensive homology over the first 23 bp (Fig. 3). This region includes the segment defined above as necessary for maximum transposition activity. Although we have analysed only one of the two extremities (IRL), the known functional similarity and comparison of the sequences (Fig. 3) suggest that the minimal sequence required at each end is of the same length. Indeed, preliminary results indicate that both extremities of IS1 exhibit similar transposition activities (P.G., D.G. and M.C., unpublished data).

The similarities and differences between the ends (Fig. 3) indicate that homology extends for ~31 bp except for a 13-bp 'insertion' immediately after position 23 in IRL. Both ends also carry four nonsense codons which prevent translation from proceeding into the element. In the case of IRL these cover all three reading frames, whereas for IRR, two of the three reading

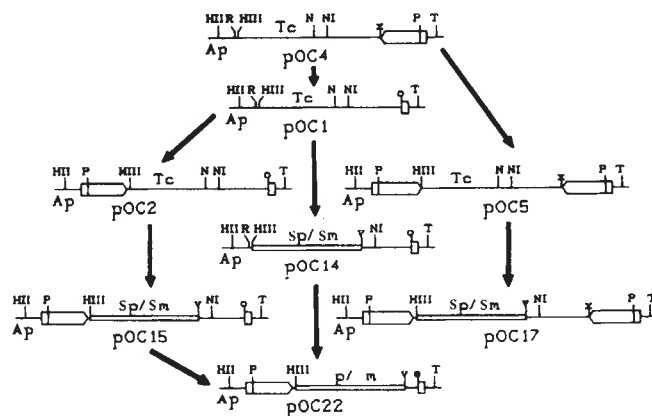


Fig. 1 Construction of IS1-flanked and end-deleted transposons. A *Bgl*II/*Xho*I fragment, carrying Tn9, was isolated from the plasmid pOX38: Tn9 (ref. 23), cloned into the unique *Pvu*II site of pBR322 (coordinate 2,067) after conversion of the recessed 3' ends to flush ends and then deleted for the internal region of Tn9 between the unique *Bst*EII sites in the flanking IS1s. The resulting plasmid, pOC4, carries a single copy of IS1. The 'pointed' extremity of IS1 is distal to the unique *Pvu*II site as depicted here, and carries IRR. Plasmid pOC1 carrying a 76-bp IRL extremity was generated from pOC4 by substitution of the DNA located between the *Eco*RI (pBR322: 0) site and the unique *Pvu*II site in IS1 with the *Eco*RI/*Pvu*II fragment of pBR322 (0-2,067). A functional IS1 was then introduced into pOC1 and pOC4 using the plasmid pR2.5IS1 (IS1 cloned into the *Eco*RI site of pBR322²³). Substitution between the *Pvu*I and *Bam*HI sites (pBR322: 3,737 and 375, respectively) of pOC1 and pOC4 by the comparable fragment of pR2.5IS1 generated plasmids pOC2 and pOC5, respectively. To avoid problems experienced with tetracycline resistance (Tc^R) selection, we substituted part of the pBR322 tetracycline gene carried by pOC1, pOC2 and pOC5 between the unique *Hind*III and *Nru*I sites (pBR322: 29 and 937, respectively) for a 1.8-kb *Hind*III/*Pvu*II fragment specifying resistance to spectinomycin/streptomycin (Sp^R/Sm^R). This generated the plasmids pOC14, pOC15 and pOC17. To generate sequential deletions of the 76-bp IS1 extremity, plasmid pOC14 was linearized at its unique *Pvu*II site and treated for various times with the exonuclease *Bal*31²⁴. The preparation was cleaved at the single remaining *Hinc*II site (pBR322: 3,908) and the fragment carrying the deleted extremity of IS1 was isolated by electrophoresis onto and elution from DEAE-cellulose paper (Schleicher & Schuell; sequences, RNA-DNA 2). Plasmid pOC15 was cleaved at its unique *Nar*I site (pBR322: 1,205), the 3' recessed end was converted to a flush end using the Klenow fragment of DNA polymerase I, the preparation cleaved with *Hinc*II, and the IS1-carrying *Hinc*II/*Nar*I fragment isolated. Following ligation of these two fragments and transformation into MC182 (*leu thr thi lac hsdR recA*), we isolated a family of plasmids, pOC22.xxx, identical except for small deletions of various sizes extending from the *Pvu*II site (defining the internal end of the IS1 fragment) towards the extremity. These are equivalent to pOC15 but carry a unique deletion between the *Pvu*II and *Nar*I sites (pBR322: 2,067 and 1,205). The extent of the deletion in 42 clones was determined initially on 5% acrylamide gels by digestion of plasmid DNA with *Bgl*I together with *Tth*III or *Rsa*I. Plasmid DNA used for sequencing was isolated from transformant clones, *Sim*I (*Ava*II)/*Tth*III fragments carrying the IS1 extremity and labelled specifically at the *Sim*I extremity with ³⁵S-TTP were subjected to chemical cleavage^{14,24}, the products separated on 10% 0.2-mm sequencing gels and visualized by autoradiography. Only selected restriction enzyme sites are included in the maps shown here. Symbols used: HII, *Hinc*II; R, *Eco*RI; HIII, *Hind*III; N, *Nru*I; NI, *Nar*I; P, *Pvu*II; T, *Tth*III; v, the hybrid *Pvu*II/*Nru*I site generated by insertion of the *Sp*/*Sm* fragment; x, hybrid *Pvu*II/*Bgl*II site generated in the cloning of the IS1; ●, hybrid *Nar*I/*Bal*31 site; ○, *Pvu*II site generated by fusion of the internal site in IS1 with that of pBR322.

frames are included. In contrast to IS10, IS50 and IS903 (refs 10–12), no sequences corresponding to the site of methylation by the *dam* methylase (GATC) are found at the ends of IS1. Both ends are known to carry promoters which direct transcription into the element¹⁵. The IRL promoter directs a transcript which covers two major reading frames in IS1, *insA* and *insB*.

An interesting feature of these extremities is that part of the sequence between the proposed -35 and -10 regions of these promoters¹⁵ exhibits extensive homology with the apparent consensus binding site of IHF (refs 16, 17). Note that a similar arrangement has been observed in the heat-shock promoter of the *rpoD* gene in *E. coli*¹⁸. The heterodimeric IHF protein, the product of the *E. coli himA* and *hip* genes^{8,9}, is involved in several site-specific recombination reactions, notably in the

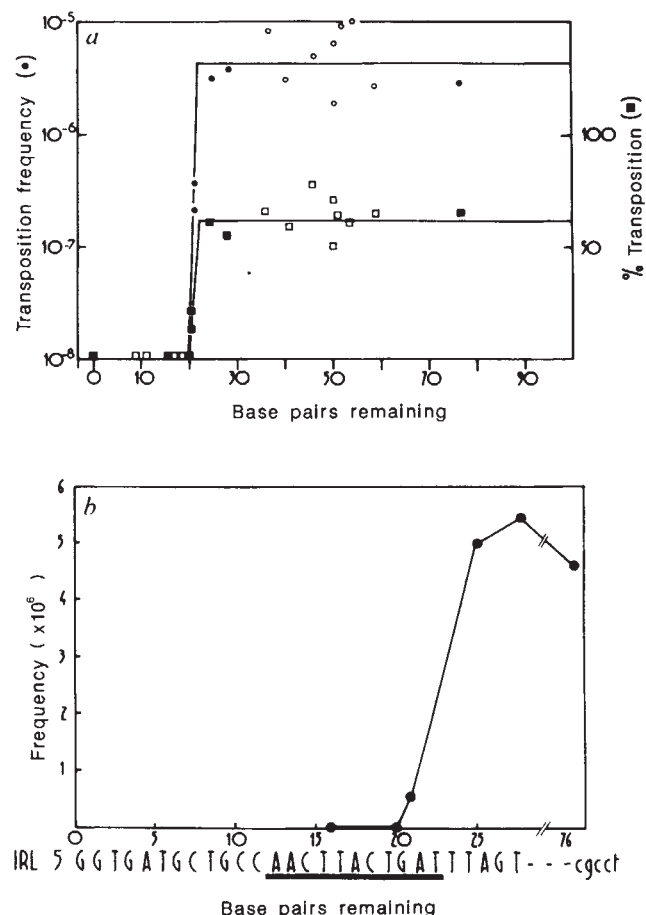


Fig. 2 Transposition frequencies as a function of the length of the deleted IS1 extremity. **a**, Overall transposition frequencies as a function of the length of the remaining IS1 extremity. The target plasmid pOX38Km was introduced into 20 representative MC182/pOC22.xxx-carrying strains and transposition assayed as described in Table 1 legend. Open symbols, the length of the remaining IS1 extremity estimated from restriction digests. Closed symbols, plasmids subjected to sequence analysis. Frequencies (○, ●) represent the results of single transposition assays for each donor strain. The data have also been expressed as a percentage of 'transposition activity' (the sum of transposition and cointransposition frequencies, □, ■). **b**, Comparison between transposition frequency and sequence. Transposition frequencies are derived from 4 independent assays of each strain. Frequencies represent the average of 4 independent transposition assays for each strain.

integration of lamboid phages^{9,17}, and controls gene expression^{9,19,20,21} at the level of transcription¹⁹ or translation²¹. The potential IHF binding site occurs at base pairs 13-23 in each extremity and ends at the point of the IRL 13-bp insertion. Comparison of these sequences with other known IHF binding sites (Fig. 3) shows that in addition to the two potential IHF sites at the extremities, a third consensus IHF site is present within IS1.

IHF or a similar protein could function directly as a cofactor in the transposition reaction (by facilitating or inhibiting interaction of the transposition enzymes with IS1), modulate the expression of IS1-encoded gene products (for example, by repressing transcription from the IRL and/or IRR promoters), or act at both levels. Recent DNA protection experiments (P.G., unpublished) indicate that purified IHF protein does indeed bind specifically *in vitro* to both extremities of IS1 and to the extremity carried by plasmids pOC22.212 (28 bp; wild-type transposition levels) and pOC22.222 (25 bp; wild-type transposition). Direct evidence for a role of IHF in IS1 transposition has, however, yet to be obtained. Our definition of the minimal

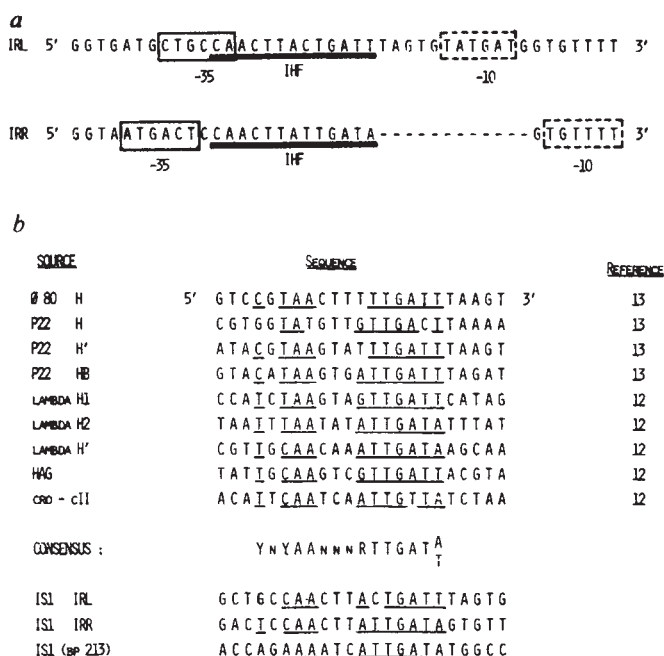


Fig. 3 Comparison of the IRL and IRR extremities of IS1 and their sequence relationship with known IHF binding sites. **a**, The sequence of the terminal 43 bp of IRL and 31 bp of IRR^{1,2} have been aligned to show their homologies. Also indicated are the relative positions of the proposed -10 (-----) and -35 (——) regions of the promoters contained within the extremities¹⁵ and the potential IHF binding sites (——). **b**, Comparison of proposed IHF binding sites in IS1 with known IHF binding sites.

sequence necessary for IS1 transposition will allow us to examine the sequence specificity at the extremities and eventually to define the proteins involved and determine the nature of their interaction.

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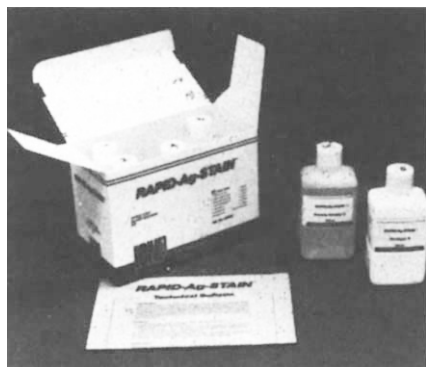
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(CBB) staining (for protein detection) and ethidium bromide staining (for nucleic acid detection), which had become the established techniques. Using Rapid-Ag-Stain the gel may be visualized in less than an hour. Sensitivity is up to 125 times CBB. The kit is suitable for proteins, RNA DNA, electrophoresis, and O'Farrel two-dimensional electrophoresis.

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THIRTY-SIX new animal viruses, Rickettsiae and antisera are available from the American Type Culture Collection, and are described in an update to the 1983 Virus Catalogue. Additions to the animal virus collection include *Ehrlichia risticii* (Potomac horse fever), two rabies viruses, several human rotaviruses and antisera, and a human cytomegalovirus. Strains are listed alphabetically by their taxonomic classification and name. Descriptive information includes original source, passage history, preparation, host of choice, incubation, effect, host range and special characteristics.

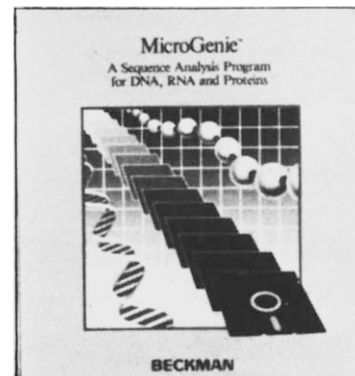
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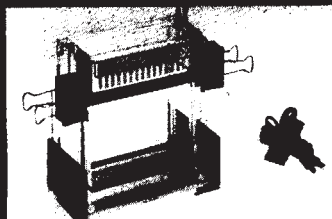
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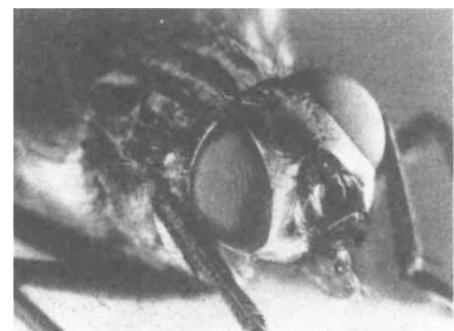


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